Chromosome Partitioning in *Escherichia coli* and Characterisation of Genes of the Fifteen Minute Region.

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To my grandparents, Fred and Mary Bull, and
the late George and Mary Addinall.

Table of Contents

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ABSTRACT

Every cell produced by the bacterial growth and division cycle must have a chromosome to be viable. Partitioning is the process which ensures this. Studies over the last thirty years have involved many attempts to identify the genetic and physical nature of the partitioning process, however the mechanism by which it works remains unclear. In this work, the polyploid nature of spherical *Escherichia coli* mutants was utilised to carry out a series of experiments designed to reveal the underlying nature of the partitioning process. The experiments show that partitioning in *E. coli* is antimitotic, that is, it actively ensures that the inevitable homozygosity of a normally unichromosomal cell is maintained, even when that cell is multichromosomal. That this was shown in spherical cells also reveals that the process is an innate property of the relationship between partitioning and division, rather than a simple consequence of separating two large chromosomes in a narrow, rod-shaped cell. This subtle, but important definition of what the partitioning process actually achieves, should be useful in further characterisation of the mechanisms involved.

Grouping of genes of similar function is a common feature of the *E. coli* chromosome. At least five clusters of genes involved in cell-wall synthesis, cell division and cell shape maintenance have been identified. The *mrd*-cluster of genes contains three implicated in morphological aspects of peptidoglycan synthesis. The presence of four uncharacterised genes and a portion of unsequenced DNA in the same region, together with proposals that the region could contain more genes involved in cell-morphology, led to the work presented here. Sequencing, followed by computer analysis and molecular-biological and genetic characterisation provided information about the transcriptional organisation of the region and the function of some of the genes. Also a new mutation affecting cell division was identified in an adjacent region of the chromosome.

ABBREVIATIONS

Cαα	- casamino acids.
cs	- cold sensitive.
DAPI	- 4,6-diamidino-2-phenylindole.
DMSO	- dimethylsulphoxide.
fts	- filamenting temperature sensitive.
HPLC	- High Pressure Liquid Chromatography.
IPTG	- isopropyl-β-D-thiogalactoside.
LRP	- leucine responsive protein.
mlt	- membrane lytic transglycosylase.
moi	- multiplicity of infection.
mRNA	- messenger RNA.
OLB	- oligo-labelling buffer.
p*[ ]*	- [ ] promoter.
PAGE	- Polyacrylamide Gel Electrophoresis.
PBP(s)	- penicillin-binding protein(s).
PCR	- Polymerase Chain Reaction.
pds	- potential division site(s).
PEG	- polyethylene glycol.
PF2K-R	- *Rattus sp. 6*-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.
PF2K-Y	- *S. cerevisiae 6*-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.
PGAM-P	- *S. pombe* phosphoglycerate mutase.
PGAM-Y	- *S. cerevisiae* phosphoglycerate mutase.
ppGpp	- guanosine tetraphosphate.
rbs	- ribosome binding site(s).
rpm	- revolutions per minute.
rRNA	- ribosomal RNA.
slt	- soluble lytic transglycosylase.
SDS	- Sodium Dodecyl Sulphate.
TEMED	- N,N,N',N', tetramethylethylethylenediamine.
Tris	- Tris(hydroxymethyl)aminomethane.
tRNA	- transfer RNA.
ts	- temperature sensitive.
Xgal	- 5-bromo-4-chloro-3-indolyl-β-D-galactoside.
$[\ ]^R$	- resistant.
$[\ ]^S$	- sensitive.

Chapter 1
Introduction

1.1 Introduction

If science is ever going to find out everything about a living thing then *Escherichia coli* will probably be that organism. Every imaginable aspect of this Gram-negative, rod-shaped, facultatively anaerobic, enteric bacterium has been and continues to be studied. As the amount of genetic, biochemical, physiological and molecular biological information available increases with phenomenal speed, the number of questions to be answered increases comparably. Therefore a point where all the diverse subjects gradually start to converge to give an overall picture, is still a long way in the future and reaching it will inevitably require even further refinements of the sophisticated techniques presently available. With the progress of genomic sequencing projects, soon every gene required for the existence of *E. coli* will be known. Then perhaps, armed with the myriad information accumulated up to that time, the pieces will gradually come together, allowing us eventually to define life itself (in one of its simplest autonomous forms at least). It appears, however that details become increasingly important as the amount of information increases and Heisenberg's uncertainty principle may apply equally well to biology as physics, in that the closer we observe, the more we distort what we are observing. The final goal may never be possible. In this laboratory even the atheists imagine God sticking his fingers into their cultures, when they get too close to the truth.

One aspect of *E. coli* which has been extensively studied is its growth and division. This rod-shaped bacterium elongates and then divides at the centre to produce two identical daughter cells. The single circular chromosome of the mother cell is replicated during this cycle and the two resulting chromosomes are distributed so that one is received by each daughter cell.

A description of this is a good example of how a superficially simple process becomes complex very quickly but also of how many different processes can be interconnected. There has been extensive study of the cell-wall, a rigid matrix of a heteropolysaccharide called peptidoglycan (or murein), and how it can be elongated and then split in two while maintaining the cell's integrity against considerable internal osmotic pressure. This wall is situated in the periplasm, that

is, between two phospholipid membrane bilayers. The cytoplasmic (inner) membrane contains proteins involved in the final stages of peptidoglycan synthesis both for elongation of the side walls and for division. Proteins involved in the initiation and progression of cell division are also associated with the cytoplasmic membrane. The outer membrane is covalently attached to the cell-wall and functions as a selectively permeable protective barrier, however it has also been found to be in association with the cytoplasmic membrane and the chromosomal replication origin, perhaps contributing to the complex cycle of events which regulate the timing and synchrony of initiation of replication. Initiation timing is closely linked to the growth and division cycle and the constant period of time required for replication of the chromosome is one of the constant parameters of the cell cycle. Distribution of chromosomes is contingent upon termination of replication and in turn links have been found between chromosomal distribution and the positioning and initiation of division septa.

The Introduction to this Thesis will try to summarise the present knowledge of this vast subject of the bacterial cell cycle. Particular emphasis will be put on studies of the distribution of chromosomes (chromosome partitioning) and on the genes involved in division and maintenance of the shape of *E. coli*, as these subjects bear most heavily on the contents of the Thesis. As emphasised above however, description of one part of this cycle is impossible without reference to many of its other aspects.

1.2 The Cell Wall

The murein sacculus, or cell wall, is responsible for the rigidly maintained rod-shape of Gram-negative bacteria such as *E. coli*. It is located in the periplasm, attached to the outer membrane (through a specific lipoprotein, Braun and Hantke, 1974, Nikaido and Vaara, 1987) and the cytoplasmic membrane (by nascent peptidoglycan). The sacculus is very strong, withstanding an internal osmotic pressure thought to be between two and five atmospheres (Mitchell and Moyle, 1956) but it is able to grow constantly throughout the cell cycle and periodically split in half to produce two cells. It predominantly consists of a monolayer of peptidoglycan, probably developing into a triple-layer at the poles, which undergoes constant turnover and recycling of precursors (Park, 1993, Wientjes *et al.*, 1991, Labishinsky *et al.*, 1991). The fundamental precursor for this massive macromolecule is a disaccharide with a pentapeptide side-chain, *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-γ-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine, which is synthesised in the cytoplasm and on the inner face of the cytoplasmic membrane (Bupp and van Heijenoort, 1993). The precursor is transported across the cytoplasmic membrane by a specific carrier molecule and incorporated into long chains of like molecules which are then cross-linked together by their peptide side-chains. The process of elongation of the chains is called transglycosylation and that of cross-linking the chains is called transpeptidation. This array of multiply cross-linked, parallel glycan strands is the sacculus.

Much information about the make up of murein has come from an analytical method called High Pressure Liquid Chromatography (HPLC, Glauner *et al.*, 1988, Kohlrausch *et al.*, 1989). This technique allows the separate identification and quantification of all the constituent parts of the peptidoglycan. For example, it can give information on the length of glycan strands, the number of peptide side-chains which are free or cross-linked, and the nature of the cross-links (these can be made between the fourth residue of each pentapeptide or between the fourth of one and the third of another). Examining the composition of the murein in wild-type cells and then comparing this with mutants or strains in which a particular protein

is overproduced, can give information about the function of the mutant gene or overproduced protein.

1.2.1 Penicillin-binding proteins

The major proteins involved in peptidoglycan synthesis belong to a family called the Penicillin-binding Proteins (PBPs). These recognise and interact with penicillin, an analogue of the D-alanyl-D-alanine dipeptide moiety of the peptidoglycan precursor (Tipper and Strominger, 1965). Exposure of isolated cytoplasmic membrane to radiolabelled penicillin and subsequent separation of proteins by SDS-PAGE has led to the discovery of eight discrete PBPs, named PBP 1A, 1B, 2, 3, 4, 5, 6, and 7 (by virtue of decreasing molecular weight). PBPs 1A, 1B, 2 and 3 are all murein synthesising enzymes, with an active site situated in the middle of the protein, and a cytoplasmic membrane anchor at their amino-terminus (Broome-Smith *et al.*, 1985, Adachi *et al.*, 1987, Edelman *et al.*, 1987). PBPs 4, 5 and 6 are hydrolytic enzymes, which have their active sites located close to their amino-terminus.

Penicillin-binding proteins 1A and 1B. The PBP1A and 1B proteins are bifunctional enzymes with transpeptidase and transglycosylase activity and so function in both the elongation and cross-linking of peptidoglycan strands. At least one of these proteins is required for viability, that is, either can be dispensed with separately but inactivation of both leads to rapid lysis (Tamaki *et al.*, 1977). They are thought to be the major peptidoglycan synthesising enzymes.

Penicillin-binding protein 2. The PBP2 protein has been shown to catalyse penicillin-sensitive transpeptidation *in vitro* and penicillin-insensitive transglycosylation in conjunction with the RodA protein (Ishino *et al.*, 1986). Inactivation of either PBP2 or its accessory protein RodA causes cells to grow as spheres (Spratt and Pardee, 1975), highlighting the role of these enzymes in production of the lateral wall of the normal rod shaped sacculus. HPLC shows no differences between the peptidoglycan composition of these spherical cells (often considered to consist entirely of polar material) and filamenting rod-shaped cells (Nanninga, 1991 and references therein);

however, other studies indicate that enzymes involved in making the lateral walls have different preferred substrates for transpeptidation reactions to those involved in septation (Begg *et al.*, 1990)[1]. Whether PBP2 is an essential protein is a matter of definition. All published literature indicates that this *is* true, with lethality being due to a block in division (Ogura *et al.*, 1989, Vinella *et al.*, 1992, Vinella *et al.*, 1993). However, the lethality can be suppressed by indirect or direct increase in the pool of guanosine tetraphosphate (ppGpp). This is thought to illustrate a coordination between ribosomal activity, growth rate and cell wall elongation by way of the stringent response (Vinella *et al.*, 1993, Cashel and Rudd, 1987). Therefore, under conditions of increased ppGpp synthesis (which include growth on minimal medium, KJ Begg personal communication), PBP2 is not essential. It is safe to say however, that PBP2 *is* essential for normal lateral cell wall synthesis, and its loss is debilitating to the extent that cells quickly accumulate suppressor mutations which raise ppGpp levels (Ogura *et al.*, 1989).

Penicillin-binding protein 3. PBP3 is a periplasmic protein anchored in the cytoplasmic membrane by its amino-terminus (Bowler and Spratt, 1989). It is directly involved in cell division, its inactivation causing cells to become filaments. That is the cells are blocked in division but capable of lateral wall synthesis (Spratt, 1975, Botta and Park, 1981, Schmidt *et al.*, 1981). These filaments eventually lyse, PBP3 being essential in all circumstances (Hara and Park, 1993). PBP3 catalyses penicillin-sensitive transpeptidation and weak transglycosylation reactions *in vitro* (Ishino and Matsuhashi, 1981) and it has been suggested that PBP3 requires an accessory protein responsible for transglycosylase activity, in a similar relationship to that between PBP2 and RodA. The only proposed candidate for this protein is FtsW (Ikeda *et al.*, 1989, Matsuhashi *et al.*, 1990), another protein implicated in cell division (Ishino *et al.*, 1989, Khattar *et al.*, 1994). The evidence for this interaction however, is entirely circumstantial and FtsW may be involved in an earlier stage of division than PBP3 (Khattar *et al.*, 1994).[2]

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[1]The genetics of cell shape maintenance will be discussed in more detail, later.
[2]The genetics of cell division will be discussed in more detail, later.

Penicillin-binding protein 4. The PBP4 protein has been shown to have [i] DD-endopeptidase and [ii] DD-carboxypeptidase activity *in vivo* (Korat *et al.*, 1990). That is, [i] it hydrolyses cross-links between glycan strands and [ii] it removes the terminal D-alanine from free pentapeptides to form tetrapeptide side-chains. It is thought to have both loosely membrane-bound and soluble forms (Mottl, 1992) and its activity (along with a penicillin-insensitive enzyme MepA) is thought to allow the insertion of new peptidoglycan and resultant expansion of the sacculus (Höltje, 1993). PBP4 is known to be responsible, in part, for autolysis of cells incubated with penicillin (*1.2.3*).

Penicillin-binding proteins 5 and 6. The PBP5 and 6 proteins share 62% sequence similarity (Broome-Smith *et al.*, 1988) and are responsible for membrane bound DD-carboxypeptidase activity (Tamura *et al.*, 1976, Amanuma and Strominger, 1980, Amanuma and Strominger, 1984). Overproduction of PBP5 causes cells to swell and become spherical and then lyse (Markiewicz *et al.*, 1985) possibly due to this enzyme reducing the amount of the preferred substrates for lateral wall extension (Begg *et al.*, 1990). Conversely a strain deleted for *dacA*, the gene for PBP5, is viable (Spratt, 1980) and accumulates these substrates (pentapeptide side-chains, Glauner, 1986). PBP6 shows neither of these features (when deleted or overproduced, Glauner, 1986, Van der Linden *et al.*, 1992). It has been proposed to be responsible for changes in stationary phase peptidoglycan (Van der Linden *et al.*, 1992) while PBP5 may stabilise the peptidoglycan by hydrolysing pentapeptide side-chains to tetrapeptides and thus limiting the amount of cross-linking (dePedro *et al.*, 1980).

Penicillin-binding protein 7. Early studies identified two very small proteins as PBP7 and PBP8, however it was subsequently found that PBP8 was a breakdown product of PBP7 (Henderson *et al.*, 1994). PBP7 has been purified and shown to be a DD-endopeptidase (Romeis and Höltje, 1994). The gene for PBP7 has been mapped to 47 minutes and sequenced. It shows similarity to known DD-endopeptidases and DD-carboxypeptidases from *E. coli* (PBPs 4, 5 and 6). The protein has

been found to bind to a major murein hydrolytic enzyme Slt70 *in vitro*, along with PBP3 (Romeis *et al.*, 1994). This provides evidence for a complex of proteins involved in division. Association of a lytic transglycosylase (Slt70), an endopeptidase (PBP7) and a bifunctional division-specific enzyme (PBP3) is an attractive idea for a division complex as it would contain activities for breaking cross-links, removing strands and then resynthesising to form the septum. Whether such a complex is linked to the proposed association of division proteins (*1.3.1*) is not known.

The genes encoding the four hydrolytic PBPs (4, 5, 6 and 7; Iwaya and Strominger, 1977, Spratt, 1980, Broome-Smith and Spratt, 1982, K Young and MF Templin, in press) can be deleted separately without loss of viability. Indeed those for PBP4, 5 and 6 can be deleted simultaneously (Edwards and Donachie, 1993, Edwards, 1994) without any serious effect, indicating a great degree of redundancy in these hydrolytic enzymes. These results also indicate that there may be as yet undiscovered DD-carboxypeptidases which can substitute for the simultaneous loss of PBPs 4, 5 and 6 and provides the tripeptide acceptor side-chains which are still detected in the triple deletion strain (Edwards and Donachie, 1993, Edwards, 1994).

1.2.2 Penicillin-insensitive peptidoglycan synthetic enzymes

A discrete period of penicillin-insensitive peptidoglycan synthesis (PIPS) during the cell cycle has been observed after initiation of septation (Nanninga, 1991). This could indicate a role for some penicillin-insensitive enzymes in the growth of the sacculus, in particular at division. Such enzymes have been identified (Keck *et al.*, 1990, Ursinus *et al.*, 1992, Hara and Suzuki, 1984). Their importance in normal cell growth remains speculative but it must be remembered that a penicillin-insensitive protein is already known to be involved in the formation of the lateral wall during normal growth (RodA); see above (*PBP2, 3*) and later (*1.4*).

1.2.3 Autolytic enzymes

Bacterial autolysis is the result of murein degradation by endogenous enzymes (autolysins) and is the reason for the lethality of antibacterial agents which target the peptidoglycan synthesis machinery. Autolysis can be induced by a number of mechanisms including inhibition of murein synthesis and subjection to chaotropic agents (Höltje and Tuomonen, 1991, Hartman *et al.*, 1974). The bacteriolytic effect of penicillin occurs in two stages (Tomasz, 1979). The first is the binding of penicillin to the murein synthetases resulting in inhibition of their activity (Blumberg and Strominger, 1974). The second is the action of autolysins and subsequent lysis of the cell wall (Tomasz, 1979, Pisabarro *et al.*, 1986). The rate of autolysis varies in direct proportion with the growth rate (Tuomonen *et al.*, 1986) and this has been related to growth rate dependant control of the stringent response (Pisabarro *et al.*, 1990). Autolysis involves the action of lytic transglycosylases and endopeptidases, enzymes which are thought normally to be involved in the reshaping of the sacculus and septum splitting (Kohlrausch and Höltje, 1991, Höltje, 1993). Autolytic enzymes have been particularly implicated in division. Cells treated with penicillin often seem to lyse preferentially at the septum (Lederberg, 1956, Mathieson, 1968, Schwarz *et al.*, 1969, Begg and Donachie, 1970) and autolysis induced by inhibition of PBP1A or B is coincident with division (Nanninga *et al.*, 1990, dePortillo *et al.*, 1989). It seems therefore that autolysis is a result of a disruption of the normal balance of synthetic and hydrolytic enzymes involved in construction of the cell wall and that that balance may be most delicate during septum formation.

Enzymes known or thought to be involved in autolysis are the lytic transglycosylases (Slts, Mlt) and endopeptidases (MepA, PBPs4 and 7); see above (Höltje and Tuomonen, 1991, MF Templin, personal communication).

1.3 Cell Division

A direct relationship between cell division and the structure and modification of the cell wall has long been proposed (Jacob *et al.*, 1963) and the identification of a peptidoglycan synthesising enzyme (PBP3) which is essential for cell division is fundamental evidence for this. It was questioned however, by the observations of Onoda *et al.* (1987) that L-forms (bacteria that have lost their cell wall) can grow and divide. Their reversion to normal bacteria showed that they retained the ability to produce a cell wall and the implication was that the cell wall was not necessary for either of these functions or, of course, for viability itself. The implications of this are that division may be an innate property of the cytoplasm and inner membrane. The possibility remains however, that since L-forms have learned to divide over a long adaptation period, and can only do so under special growth conditions, they may have developed alternative mechanisms to those which manage and regulate division in normal bacteria (Gumpert, 1992).

1.3.1 Division proteins

The study of division has been based on the isolation of conditional mutants which can not divide at the non-permissive temperature. These filamentous temperature sensitive (*fts*) strains are affected in one of a number of different genes which appear to act sequentially in the division process. The genes have been mapped and sequenced and in most cases the proteins and their roles in cell division have been extensively studied. The genes for six of these proteins lie in a large cluster of genes for division and cell wall synthesis proteins, at 2.5 minutes on the chromosomal map (Donachie, 1993). Clusters of genes with functions related to cell division, morphology and peptidoglycan synthesis have been found at various positions on the chromosome. These clusters have been named *mra* (2.5 minutes), *mrb*, *mrc etc* with the *mr* coming from the name **m**urein **r**egion.

FtsZ. The FtsZ protein has been identified as being required for the earliest step in cell division (Lutkenhaus *et al.*, 1980, Begg and Donachie, 1985). It is the most abundant cell division protein,

estimated to be present in 5,000 to 20,000 copies per cell (Bi and Lutkenhaus, 1991). The *ftsZ* gene is located in the *mra* cluster. FtsZ is a cytoplasmic protein but has been shown to aggregate, to form a circumferential ring on the inner face of the cytoplasmic membrane, prior to division. It then remains localised at the leading edge of the division septum as constriction takes place (Bi and Lutkenhaus, 1991, Lutkenhaus, 1993). The protein has four regions of homology with eukaryotic tubulins and has been demonstrated to have weak GTPase activity (RayChaudhuri and Park, 1992, deBoer *et al.*, 1992, Mukherjee *et al.*, 1993). These results have lead to the current view that FtsZ forms a contractile ring around the cell which gradually constricts the cytoplasm at the division site and is closely followed by the leading edge of septal peptidoglycan synthesis. Such a cytokinetic ring would explain the ability of L-forms to divide without a cell wall (Onoda *et al.*, 1987)

Highlighting its importance for division, FtsZ is the target for specific division inhibitors of different origins (Bi and Lutkenhaus, 1993). As part of the cellular response to DNA damage, the SOS response, production of the SulA protein is induced (Huisman and D'Ari, 1981). This protein interacts directly with FtsZ to prevent formation of the FtsZ ring (Bi and Lutkenhaus, 1993). The MinC and MinD proteins act together to inhibit cell division with an accessory protein, MinE, being responsible for releasing the inhibition at the cell centre, to allow normal division. Inactivation of MinE causes filamentation, whereas inactivation of MinC or MinD causes production of minicells (and short anucleate rods in some alleles, Jaffé *et al.*, 1986), the cells being able to divide at sites other than the centre (deBoer *et al.*, 1992). The MinCD inhibitor was also shown to prevent formation of the FtsZ ring (Bi and Lutkenhaus, 1993) and the isolation of FtsZ mutants resistant to the action of both SulA and MinCD, indicates strongly that both inhibitors act in the same way (Bi and Lutkenhaus, 1990b). Minicell-producing mutants divide only once per generation and so result in a heterogeneous population of cells with respect to their length. That is, the divisions which produce minicells are *instead of* normal divisions (Teather *et al.*, 1974). Overproduction of FtsZ produces a subtly different minicell phenotype in that cells can divide at both the cell centre and the

poles in the same generation (Bi and Lutkenhaus, 1990a). FtsZ can therefore increase the number of division events and remains the only protein known to be able to do so.

FtsA. FtsA is a 46.4 kD protein whose gene is located directly upstream of *ftsZ.* Only 150 molecules of FtsA are estimated to be present in the cell (Wang and Gayda, 1992) and the ratio of FtsA to FtsZ has been proposed to be critical for division to proceed (Dai and Lutkenhaus, 1992, Dewar *et al.*, 1992). The protein localises to the cytoplasmic membrane but has no signal sequence or exceptional hydrophobicity, possibly indicating an interaction with other membrane proteins (Chan and Gayda, 1988, Pla *et al.*, 1990). It has been reported that temperature sensitive *ftsA* and *ftsI* (PBP3) alleles can be suppressed by overproduction of a protein called FtsN (Dai *et al.*, 1993). These observations have been proposed to indicate that PBP3 and FtsA may interact. The characteristic phenotype of FtsA mutants is of long filamentous cells with regular, blunt constrictions, in contrast to cells blocked by inactivation of PBP3 which produce straighter filaments. It is suggested that the FtsA mutant cells are blocked at a later stage of division than the PBP3 mutant cells (Begg and Donachie, 1985), that is, FtsA may not be required for the initiation of PBP3 activity but possibly for its continuation. This could be explained if FtsA was only required for later stages of septal formation, however re-evaluation of some FtsA mutants has shown that their phenotypes are leaky (that is, some FtsA activity remains) and experiments to inactivate the gene completely have suggested that the true FtsA null phenotype may be straight-sided *and* constricted filaments (Hale *et al.*, 1994). If this turns out to be the case then previous results could be interpreted to mean that more FtsA protein (or activity) is required for later stages of division than early stages but Hale *et al.* (1994) have shown that at least some is required at all times. The same group has provided evidence of direct interaction between FtsA and FtsZ (Hale *et al.*, 1994) and it has been suggested that the similarity of FtsA to a class of ATPases (Bork *et al.*, 1992) could indicate a role in mediating the GTPase activity of FtsZ (Hale *et al.*, 1994).

FtsQ. FtsQ is a 31 kD protein that is essentially periplasmic but includes a membrane spanning domain and a short cytoplasmic region (Begg *et al.*, 1980, Yi and Lutkenhaus, 1985). It shares this structure and a non-cleavable sequence near its amino terminus with FtsL, PBP3 and FtsN (Dai *et al.*, 1993). The protein is estimated to be present in only 50 to 100 copies per cell (Carson *et al.*, 1991). The *ftsQ* gene lies directly upstream of *ftsA* and *ftsZ* (Lutkenhaus and Wu, 1980). Inactivation of this essential protein produces straight aseptate filaments (Carson *et al.*, 1991); however, when combined with a *ts* spherical mutation it shows constrictions (Begg and Donachie, 1985) and has been suggested to block division between the activities of FtsZ and FtsA (Begg and Donachie, 1985).

Penicillin-binding protein 3 (FtsI). The *ftsI* gene lies in the two minute (*mra*) cluster (approximately 11 kb upstream of the *ftsQ*, *A* and *Z* genes) and encodes the septum specific murein synthetase PBP3 (Ishino and Matsuhashi, 1981, Spratt, 1977). PBP3 is estimated to be present in 50 copies per cell (Spratt, 1977) and is a transmembrane protein with a catalytic domain located in the periplasm (Bowler and Spratt, 1989). Its peptidoglycan synthesising activity has been discussed above (*1.2.1*). Inactivation of *ftsI* or inactivation of the PBP3 protein itself (for example, with PBP3 specific antibiotics) produces filaments which can be shown to be constricted by combination with a spherical mutation (Begg and Donachie, 1985).

FtsL. The *ftsL* gene, also called *mraR* (Ueki *et al.*, 1992), lies directly upstream of *ftsI* and is the site of the *ftsI36* and *lts36* mutations (Matsuhashi *et al.*, 1990). The 13.6 kD FtsL protein is present in 30 to 40 copies per cell, and mutants in the gene produce long aseptate filaments (Guzman *et al.* 1992). The similar membrane topology, mutant phenotype and number of proteins per cell of FtsL, PBP3 and FtsQ has lead to the proposal that these three proteins may form a 'stoichometric complex' (Guzman *et al.* 1992; also see FtsN, below). It is also interesting that the FtsL protein contains a potential leucine zipper motif in its periplasmic domain which could be used in dimerisation (Guzman *et al.*, 1992).

FtsW. The *ftsW* gene, situated in the centre of the *mra*-operon was identified on the basis of a filamentous mutant with some unusual properties. The filamentation only occurred at certain cell densities at the non-permissive temperature (Ishino *et al.*, 1989, Ikeda *et al.*, 1989, Matsuhashi *et al.*, 1990). On the basis of this phenotype and significant sequence similarity to the RodA protein it was proposed that FtsW might act as an accessory protein to PBP3. The homology to RodA suggests that FtsW too is a membrane protein with ten membrane-spanning segments (Matsuhashi *et al.*, 1990, Matsuzawa *et al.*, 1990, MM Khattar, unpublished). Recent work (Khattar *et al.*, 1994) has involved identification of the *ftsW* gene product, FtsW, which shows aberrant behaviour in SDS-PAGE in the same way as RodA (Stoker *et al.*, 1983b). Also, a newly isolated *ftsW* mutant exhibits density independent filamentation (Khattar *et al.*, 1994) and when combined with spherical mutants (Begg and Donachie, 1985) shows morphological characteristics indicative of an early role in the division process (Khattar *et al.*, 1994).

EnvA. EnvA is a 34 kD cytoplasmic protein encoded by the final gene in the *mra*-operon. It is followed by a strong transcriptional terminator (Beal and Lutkenhaus, 1987). Deletion or overexpression of *envA* is lethal (Sullivan and Donachie, 1984, Beal and Lutkenhaus, 1987) and a mutation (*envA1*) produces characteristic chains of cells blocked in septum splitting (Normark, 1970, Normark *et al.*, 1969) when grown in rich media. The phenotype is pleiotropic, with the outer membrane being hyperpermeable to some antibiotics (Normark, 1970, Grunstrom *et al.*, 1980). Recently, it has been reported that the EnvA protein has UDP-3-*O*-acyl-N-acetylglucosamine deacetylase activity and therefore is directly involved in cell wall metabolism (Young *et al.*, 1994). Although the mutant can form long chains of cells, the formation of the septum is not affected and it is thought that the only division defect is a slowing in covalent detatchment of the two newly formed cells (Wolf-Watz and Normark, 1976). As such, the mutant is strictly defective in cell separation rather than septation.

cha. This is an unsequenced mutation located at 73 minutes (KJ Begg, unpublished). Any protein product associated with the defect remains to be discovered. *cha* is a conditional mutant with a similar phenotype to the *envA1* mutant at the non-permissive temperature (Donachie *et al.*, 1984). That is, it forms chains and has outer membrane permeability defects (Chakraborti *et al.*, 1986). There is further evidence that the *cha* defect is similar to that in *envA1* in that the lethality of *cha* is complemented by the *envA* gene in single copy (KJ Begg, unpublished). *envA* and *cha* are also discussed in Chapter 5 (*5.4.5*).

FtsK. A division mutant at a locus called *ftsK* has recently been mapped to 20 minutes on the chromosome and sequenced. The predicted FtsK protein has a molecular weight of ~80000 kD. The *ftsK* gene lies downstream of an SOS dependent promoter in an operon with the gene encoding leucine-responsive regulatory protein (LRP) (SJ Dewar, KJ Begg and WD Donachie, unpublished). From combining the mutation with a spherical mutation, as described in Begg and Donachie (1985), *ftsK* has been implicated in very late stages of septation (KJ Begg, unpublished). Such experiments must now be treated with caution as the possibility remains that leaky alleles could give filaments with constrictions, in contrast to a total null mutation; see FtsA, above. Interestingly the *ftsK* defect can be suppressed totally by combining it with a deletion of *dacA*, the gene for PBP5 (KJ Begg, unpublished).

FtsN. The gene *ftsN* was isolated as a multicopy suppressor of *ftsA* mutants and was subsequently found to suppress *ftsI* mutations in the same way (Dai *et al.*, 1993). It lies at 88.5 minutes on the chromosome and encodes a 36 kD protein, FtsN. Inactivation of FtsN produces long aseptate filaments. The protein is estimated to be present in 50 copies per cell (Dai *et al.*, 1993). As a consequence of these two facts together with FtsN's similar membrane topology to the same proteins, it has been put forward as another component of the hypothetical 'stoichometric complex' involved in septum formation (Lutkenhaus, 1993).

FtsY, FtsE, FtsX. The *ftsY*, *ftsE* and *ftsX* genes form an operon at 76 minutes on the chromosome (Gill *et al.*, 1986). FtsE has homology to nucleotide binding proteins involved in protein transport (Gibbs *et al.*, 1992) and the filamentous phenotype of some *ftsE* mutants is growth rate dependent (Taschner *et al.*, 1988). FtsY is homologous to a eukaryotic secretory protein (Gill and Salmond, 1990), while the product of *ftsX* has no significant sequence similarity to known proteins. All three proteins lack signal sequences (Gill *et al.*, 1986) but are associated with the cytoplasmic membrane (Gill and Salmond, 1987). It has been suggested (e.g. Donachie *et al.*, 1984) that these proteins are involved in the transport of other essential cell division proteins through the cytoplasmic membrane, hence the filamentous phenotype of *ftsYEX* mutations.

Penicillin-binding protein 2. The list of division proteins should perhaps formally include PBP2, because it has been proposed to be a positive regulator of cell division (Vinella *et al.*, 1990, Vinella *et al.*, 1992, Vinella *et al.*, 1993) however this has been described briefly above (*1.2.1*) and will also be discussed in a later section (*1.4*).

1.3.2 Division regulation

The regulation of cell division proteins is just beginning to be elucidated. Examples of transcriptional, translational and functional regulation exist. Most studies to date have concerned the cell division genes of the *mra*-operon and particularly *ftsZ.*

The *mra*-operon consists of 16 open reading frames of which the protein products of 15 have been identified. The open reading frames share the same transcriptional orientation and either overlap or are separated by only a few bases. Of the genes whose functions have been discovered, those which are not cell division proteins as described above, are all involved in stages of peptidoglycan precursor synthesis. The order of the genes is *mraZ* (function unknown), *mraW* (essential, but no reported division phenotype, Carrión *et al.*, 1994), *ftsL* (*mraR*), *ftsI* (*pbpB*), *murE*, *murF*, *mraY* (no protein identified but probably essential, D Boyle, unpublished), *murD*, *ftsW*, *murG*, *murC*, *ddlB*, *ftsQ*, *ftsA*, *ftsZ*, and *envA* (Donachie, 1993). Several promoters have been identified in the region but only one transcriptional

terminator (Beall and Lutkenhaus, 1987). This lies distal to *envA* and marks the end of the operon. The structure of the operon therefore suggests that it is highly likely that [i] many of these genes are cotranscribed and that [ii] many of the genes are translated from more than one species of mRNA. The control of gene expression from this region is, not surprisingly, complex.

Transcriptional regulation. Seven distinct promoters capable of transcribing *ftsZ* have been located between *ddlB* and *envA* (Donachie, 1993). The strengths of these promoters vary and some have been proposed to modulate FtsZ expression in response to growth rate (Donachie *et al.*, 1984, Dewar *et al.*, 1989, Aldea *et al.*, 1990, Smith *et al.*, 1993), indeed, one of the promoters in *ddlB* has a 'gearbox' consensus which is characteristic of several other genes that respond inversely to growth rate (Aldea *et al.*, 1990). As growth rate falls, cell volume decreases because of an increased rate of cell division per unit mass. This ensures that a maximum number of individual cells (per total mass) enters the 'survival' stage of bacterial life called stationary phase. This is important as 90–99% of cells die after prolonged stationary phase (Kolter *et al.*, 1993). It has been suggested that growth rate dependent promoters in the *mra*-operon are required to increase the rate of transcription of cell division genes during this process (Donachie *et al.*, 1984) such that increased amounts of cell division proteins per unit mass would allow the increased division. [That is, if expression of division genes were linked solely to volume and division required the production of quantal 'packets' of division proteins (Teather *et al.*, 1974), then cells would not get smaller at lower growth rates.] Actual measurement of the amounts of FtsA and FtsZ, however have been reported to remain at a constant concentration independent of growth rate (Wang and Gayda, 1992, Bi and Lutkenhaus, 1993). One reason for this anomaly may be that 80% of *ftsZ* transcription actually comes from a different promoter which has been shown to oscillate in a cell cycle dependant manner (Garrido *et al.*, 1993), compared to only 10% from the 'gearbox' promoter (Ballesteros *et al.*, 1994). The periodic feature of *ftsZ* expression is thought to allow the production of a threshold amount of FtsZ at a specific time during the cell cycle (Garrido *et al.*,

1993). Expression of *ftsZ* from one of the promoters internal to *ddlB* has been shown to be positively regulated by the *sdiA* gene product (Wang *et al.*, 1991). It is unclear whether this regulation is important because, although overexpression of *sdiA* causes an increase in expression of *ftsZ*, its deletion has no obvious effect. It is thought that many of these instances of apparently peripheral control could be fail-safe mechanisms that avoid catastrophes under stress conditions (Ballesteros *et al.*, 1994). Experiments where the level of *ftsZ* expression has been artificially increased or lowered (Ballesteros *et al.*, 1994, Tétart *et al.*, 1992) seem to show that, although division can still take place at a variety of concentrations, this sort of disruption causes cells to divide at the wrong length. Thus the overall regulation of expression of cell division genes may be critical for correct coordination between growth and division, with FtsZ concentration as the limiting factor.

Translational regulation. Normal expression of both *ftsZ* and *ftsI* appears to require distant upstream regions of the *mra*-operon (Bi and Lutkenhaus, 1991, Hara and Park, 1993), possibly indicating the production of a single 18 kb transcript of the entire *mra*-operon (Hara and Park, 1993). In such an event the production of vastly different comparative levels of the division proteins FtsQ (25 molecules per cell), FtsA (150), FtsZ (20000), PBP3 (50) and FtsW (very low but not yet calculated, MM Khattar, unpublished) from the same transcript must be due to post-transcriptional control. Differential translation of *ftsQ, A* and *Z*, has been shown from the same transcript (Mukherjee and Donachie, 1990). Poor translation also appears to be the reason for low levels of PBP3 and FtsW (MM Khattar and G Roberts, unpublished). Other workers have shown that mRNA from which FtsZ and FtsA are produced is processed by RNase E, resulting in stabilisation of the *ftsZ* part of the transcript and destabilisation of the *ftsA* part (Kam *et al.*, 1994). This could be a further factor in the differential expression of these genes.

A different form of post-transcriptional control is the presence of a promoter and open reading frame which would produce a small 'antisense' RNA complementary to the 5' end of the *ftsZ* mRNA (Dewar and Donachie, 1993). This promoter has been shown to be active and

has an associated terminator. Also its presence in high copy number at high temperatures causes a block to cell division (Dewar and Donachie, 1993). If proven to be active in single copy on the chromosome this could act in an identical way to the antisense RNA produced from the relict bacteriophage gene *dicF* (e.g. Tétart *et al.*, 1992), to control the level of *ftsZ* expression.

Functional regulation. How the septum specific activity of PBP3 is activated is not clear. It has been reported that PBP3 is not enriched in minicell membranes (Buchanan, 1981)[3]. Therefore the subcellular location of PBP3 appears not to change at division. It has also been demonstrated that the normal C-terminal processing of PBP3 by the *prc* product is not essential for its function (Hara *et al.*, 1991). It is not known whether concentration of PBP3 are cyclically controlled but it has been suggested that the supply of murein precursors may regulate PBP3 activity (Markeiwitz *et al.*, 1982, Pisbarro *et al.*, 1986). PBP3 has also been proposed to interact with FtsA (Tormo *et al.*, 1986) and FtsZ (Ayala *et al.*, 1988), possibly forming a functional complex (Nanninga, 1991, Guzmann *et al.*, 1992, Pla *et al.*, 1993). PBP3 may therefore be regulated by the assembly and activity of other members of such a complex. An additional feature to the regulation of PBP3 is the repression of *ftsI* by the MreB protein (Doi *et al.*, 1988). The gene for this protein forms part of the *mre*-operon, at 71 minutes, which includes *mreB*, *mreC* and *mreD* (Doi *et al.*, 1988). While MreB is responsible for negative regulation of PBP3 (Wachi and Matsuhashi, 1989), a deletion covering all three *mre* genes produces increased levels of PBP1B and PBP3. MreB is a cytoplasmic protein that has sequence similarity to FtsA (Matsuhashi *et al.*, 1990) and shows structural similarity to the same group of ATPase proteins as FtsA (Bork *et al.*, 1992). Since FtsA has been suggested to have a regulatory role in division (Tormo *et al.*, 1986, Donachie *et al.*, 1984), probably through physical interaction with FtsZ (Hale *et al.*, 1994), a similar interaction between MreB and PBP3 could therefore be possible. The inhibition of FtsZ by interaction with SulA or MinCD proteins is discussed above (*1.3.1*) and illustrates another mode of

[3]Minicells are often percieved as consisting solely of septal material

functional regulation of division. FtsZ appears to be under yet another level of control in that it requires activation (by potassium ions or by high concentrations of itself) before it exhibits GTPase activity. This activity is assumed to be required for the assembly of the FtsZ ring (deBoer *et al.*, 1992, Mukherjee *et al.*, 1993).

The regulation and functioning of the other known cell division proteins and their genes is not well understood.

1.4 Cell Shape

The regulation of cell shape is clearly closely related to the synthesis and modification of the cell wall (*1.2*), as it is the cell wall which determines the shape. This section concentrates on genes or proteins which have been implicated specifically in determining the rod-shape of *E. coli*.

In a steady-state culture, the rod-shape of *E. coli* changes by elongation of the lateral wall (and division) with the width of the cell staying approximately constant. Over a range of growth rates however, both the width and the average length of cells change, such that the length-to-width ratio (or shape factor) is very similar, regardless of growth rate (Zaritsky, 1975). At very slow growth rates this constant factor is lost, and cells become much shorter and fatter (Zaritsky *et al.*, 1993). The constant shape factor is an important aspect of the general coordination between cell mass and initiation of replication (Cooper and Helmstetter, 1968, Donachie, 1968), cell length and chromosome partitioning (Donachie and Begg, 1989) and chromosome and septum positioning (Mulder and Woldringh, 1989); see later.

When discussing morphological aspects of bacteria one must distinguish therefore between the normal rod shape - a right circular cylinder with hemispherical polar caps, and the shape factor - the length-to-width ratio of a rod. Morphological genes or proteins discussed in this section, are those which have been shown to cause a cell to assume a shape other than that of a rod, either by their mutation, overexpression or absence. Factors which alter the shape-factor and not the shape, seem to exert their influence by way of the general coordination of growth, division, replication and morphology mentioned above and in section 1.3.2. For example: starvation causes cells to get very short as they enter stationary phase (Kolter *et al.*, 1993); *dam*$^-$ cells are bigger than their *dam*$^+$ counterparts, probably due to partial induction of the SOS response (Vinella *et al.*, 1992b, Addinall and Donachie, unpublished); and cell width and septum position can be influenced by the activity of adjacent nucleoids (Woldringh *et al.*, 1994, Mulder and Woldringh, 1989).

1.4.1 Proteins and genes involved in cell shape

A major factor in the identification of proteins involved in the maintenance of the bacterial rod shape has been the use of the amidino-penicillin, mecillinam (Greenwood and O'Grady, 1973). It was found that incubation of cells with this compound caused them to become spherical (Melchior *et al.*, 1973, James *et al.*, 1975, Spratt and Pardee, 1975) and that selection for resistance to mecillinam produced a high proportion of spherical mutants (Matsuhashi *et al.*, 1974, Iwaya *et al.*, 1978). That is, these cells grew and divided as spheres, rather than rods. The characterisation of mecillinam action, mecillinam resistant mutants and spherical strains isolated by other methods (Adler *et al.*, 1968, Henning *et al.*, 1972, Matsuhashi *et al.*, 1974, Matsuzawa *et al.*, 1973, Westling-Häggström and Normark, 1975) has identified a number of proteins and genes involved in maintenance of cell-shape. These can be divided roughly into three groups. [i] Inactivation of PBP2 or RodA gives spherical cells which have no potential for elongation, that is, if division is inhibited in these mutants, they remain spherical. These genes are thought to be absolutely required for cell elongation. [ii] *cya*, *crp* and *mre* mutations produce rounded cells which retain potential for elongation, that is, subsequent division inhibition causes them to filament (Donachie *et al.*, 1984). [iii] Over-expression of either the *dacA* gene (encoding PBP5) or the *bolA* gene causes cells to grow as spheres.

Penicillin-binding protein 2 and RodA. The genes for PBP2 and RodA were originally identified as one shape-determining locus in the fifteen minute region of the chromosome (e.g. Iwaya *et al.*, 1978). It was subsequently found that there were two types of spherical mutants which mapped to this region (Tamaki *et al.*, 1980, Spratt *et al.*, 1980). In one type, PBP2 was shown to be temperature sensitive, that is, at the non-permissive temperature, PBP2 could not be labelled by radioactive penicillin. In the other type of mutant, PBP2 behaved normally in labelling experiments (Tamaki *et al.*, 1980, Spratt *et al.*, 1980). The involvement of PBP2 in cell shape maintenance had been expected because of experiments using radioactive mecillinam, showing that mecillinam caused cells to become spherical by binding very specifically to PBP2 (Spratt, 1975,

Spratt, 1977). The identification of another locus next to the gene for PBP2, also responsible for cell shape maintenance was an indication of the presence of a morphological gene cluster. This cluster has been given the name *mrd* (Tamaki *et al.*, 1980)[4], and also contains the gene for PBP5 (*dacA*).

PBP2 has been discussed previously with respect to its murein synthetic activity (*1.2.1*) and its proposed role in cell division (*1.3.1*). It is thought to act in tandem with RodA to provide the peptidoglycan synthetic activity responsible for lateral cell elongation which consequently maintains the rod shape (Ishino *et al.*, 1986, Matsuzawa *et al.*, 1990). D'Ari and co-workers (Ogura *et al.*, 1989, Vinella *et al.*, 1990, Vinella *et al.*, 1992, Vinella *et al.*, 1993) maintain that PBP2 is essential for division as well as lateral elongation. The 'lethality' of PBP2 mutations has been discussed previously (*1.4.1*) and Vinella *et al.* (1993) have shown it to be reversed by overexpression of *ftsZ*, indicating that the division apparatus may be limiting in these spherical cells. This result has also been disputed (KJ Begg, personal communication; see also Chapter 3, *3.3.2*). Suppressors of the lethality of *pbpA* deletions and mutations arise specifically in genes which, when mutated, cause an increase in the levels of ppGpp. Normally ppGpp levels are inversely correlated with growth rate (Cashel and Rudd, 1987). This nucleotide is the effector of the stringent response and an increase in its level alters the affinity of RNA polymerase for a large set of promoters. In this way, the stringent response is thought to adjust the ribosome concentration and translation capacity to aminoacyl-tRNA availability and growth rate (Cashel and Rudd, 1987). The suppression of PBP2 mutants by high levels of ppGpp or overproduction of FtsZ has therefore been proposed to reveal a coordination between cell division, elongation and growth rate. Therefore a positive effect on septation by ppGpp, as part of the stringent response has been postulated (Vinella *et al.*, 1993).

RodA is a cytoplasmic membrane protein with ten membrane-spanning segments and can be cross-linked to PBP2 in the

[4]The genes for PBP2 and RodA have two names because of this nomenclature. Thus PBP2 is encoded by *pbpA* or *mrdA* and *RodA* is encoded by *rodA* or *mrdB*. To avoid confusion, the former names are used in this thesis, however the group of genes is still called the *mrd*-cluster or operon.

membrane (Matsuzawa *et al.*, 1990). An interaction between RodA and PBP3 has also been proposed (Begg *et al.*, 1986) due to the mutual suppression of RodA amber and *ftsI ts* mutations. This suppression was found actually to be due to an increase in the amounts of PBP2 and PBP5, caused by the RodA amber mutation (Begg *et al.*, 1990), suggesting a more subtle interaction between the elongation and septation processes, based on control of the levels of preferred murein substrates for the enzymes involved in each process (Begg *et al.*, 1990; see 1.2.1, *Penicillin-binding proteins 5 and 6*). The mechanism by which an amber mutation in *rodA* causes elevated expression of genes both downstream and upstream, is unknown. RodA mutations have also been proposed to be lethal (Vinella *et al.*, 1990), however this appears not to be the case (Spratt, 1980, KJ Begg, unpublished). Nevertheless, such mutants are prone to accumulate the same suppressors which arise in PBP2 deficient cells (Spratt, 1980, Vinella *et al.*, 1990, this work - *3.3.1*).

cya, crp and mre mutations. Mutations in *cya* (the gene for adenyl cyclase at 85 min on the chromosome) and *crp* (the gene for cyclic AMP receptor protein, CAP, at 74 min) give rise to mecillinam resistant, rounded cells (Aono *et al.*, 1979, Kumar, 1976). The shape of the former two is highly dependent on growth conditions and cell density and their importance in cell shape determination is doubtful (WD Donachie, personal communication). It has been found that the strains mutated in *cya* and *crp* have a higher frequency of dividing cells than normal, leading to the suggestion that the cAMP-CAP complex exerts a negative regulation on septal synthesis (Donachie *et al.*, 1984). [The shape of these mutants could therefore be due to a change in the shape factor.] Observations of minicell mutants showed that the cAMP-CAP complex was required for divisions which produced anucleate rod-shaped cells but not for those which produced minicells (Jaffé *et al.*, 1988). The significance of this is not understood.

The *mre* cluster of genes lies at 71 min on the *E. coli* chromosome. This was identified as a locus (envB) involved in maintenance of cell shape by Westling-Häggström and Normark (1975). It is now known that there are at least five genes in this

region, *mreBCD-orfE-orfF*(*cafA*), and that loss of *mreB* or *mreBCD* results in cells becoming rounded (Wachi *et al.*, 1987, Wachi *et al.*, 1989, Wachi *et al.*, 1991). It was shown that overexpression of *mreB* has a negative effect on *ftsI* expression (and consequently on the concentration of PBP3) causing the formation of filamentous cells; conversely, in *mreB* mutants and an *mreBCD* deletion, the levels of both PBP3 and PBP1B are increased and cells are spherical (Wachi *et al.*, 1987, Wachi and Matsuhashi, 1989). These results and the significant homology of MreB to FtsA (Doi *et al.*, 1988), have led to the proposal that these are both regulatory proteins, with opposing functions to ensure that rod shaped cells begin to elongate or divide at the correct position and time (Wachi and Matsuhashi, 1989). There is a cluster of genes in *Bacillus subtilis* which is highly homologous to the *mre* cluster of *E. coli.* Mutations in the *B. subtilis mreD* homologue, cause cells to grow as spheres, indicating that *mreB* is not the only cell shape regulation gene in that operon (Varley and Stewart, 1992).

Other situations found to produce spherical cells. When an *lpp* mutation is combined with an *ompA* mutation the resultant cells grow as spheres rather than rods (Sonntag *et al.*, 1978). *lpp* encodes Braun's lipoprotein which is responsible for attachment of the outer membrane to the cell wall (Braun and Rehn, 1969), and *ompA* encodes a major outer membrane protein which has been shown to be concentrated predominantly at cell poles and nascent septa (Begg, 1979). This seems to indicate that an interaction between the cell wall and the outer membrane is required for maintenance of cell shape, however neither mutation by itself has a similar effect (Donachie *et al.*, 1984).

Overproduction of the PBP5 protein by increased copy number of the *dacA* gene initially causes cells to grow as spheres and ultimately causes them to lyse. The spherical aspect of this phenotype has been attributed to the elevated levels of PBP5 leading to a surfeit of tetra- or tripeptide side chains in the peptidoglycan. The tripeptide side-chains are thought to be the preferred substrate of the septum synthesising enzymes as opposed to the lateral wall synthesising enzymes, thus resulting in overproduction of septal

peptidoglycan - hence the cells are round (Markeiwitz *et al.*, 1982, Begg *et al.*, 1990).

Overexpression of the *bolA* gene also causes cells to grow as spheres (Aldea *et al.*, 1988). The *bolA* gene is under the control of a 'Gearbox' promoter (Alea *et al.*, 1989), causing its expression to be inversely proportional to growth rate. It has therefore been suggested that the product of the *bolA* gene is required to ensure the higher relative rate of division during entry into stationary phase. Thus overproduction of the protein would result in extra divisions ultimately giving a shape factor of 1 - a sphere. Since some division genes have been shown to be, at least partially, under 'Gearbox' promoter control themselves (*1.3.2*), the role of *bolA* is uncertain, furthermore, it can be deleted with no significant effect on growth or division of cells.

1.4.2 The mrd-cluster of genes

The three genes implicated in cell shape control (*pbpA*, *rodA* and *dacA*), within 6 kb of one another, at 15 minutes on the chromosome, comprise the *mrd*-cluster. Analysis of proteins produced from the region and subsequent sequencing, revealed that there were at least nine genes in the region. Five have now been characterised: *leuS*, which encodes leucyl tRNA synthetase, *holA*, which encodes the delta subunit of DNA polymerase III holoenzyme, *pbpA*, *rodA* and *dacA*; the remaining four have been shown to produce proteins (Low *et al.*, 1971, Spratt *et al.*, 1980, Stoker *et al.*, 1983a, Stoker *et al.*, 1983b, Asoh *et al.*, 1986, Takase *et al.*, 1987, Matsuzawa *et al.*, 1989, Dong *et al.*, 1993). Two of the uncharacterised genes (*rlpB* and *rlpA*) were shown to encode **r**are **l**ipo**p**roteins, with signal sequences targeting them to the cytoplasmic membrane and it was proposed that these might be involved in peptidoglycan modification (Takase *et al.*, 1987). An unsequenced region between *leuS-rlpB-holA* and *orf(ybeB)-orf(ybeA)-pbpA-rodA-rlpA-dacA* was thought not to contain any genes on the basis of radioactive protein-labelling experiments with fragments of DNA from the region (Takase *et al.*, 1987). Further characterisation of the genes of this region and their transcriptional organisation makes up a large portion of this Thesis.

1.5 Chromosome Replication

The major genetic element in *E. coli* is a circular, double-stranded, DNA chromosome. In order to survive, the bacterium must replicate this chromosome once per cell cycle and then ensure that one copy of the chromosome ends up in each of the two cells produced at division. The replication of the chromosome has been studied extensively and the components of the replication apparatus are well understood (Masters, 1989). The chromosome is replicated bi-directionally (eg. Masters and Broda, 1970, Prescott and Kuempel, 1972) from a fixed origin, by DNA polymerase III holoenzyme, which takes a fixed amount of time to do so at most growth rates (approximately 40 min at 37°C, Cooper and Helmstetter, 1968). This is one of the few constants in the bacterial cell cycle and other processes like cell division and chromosome partitioning must be coordinated in order to accommodate this constant. In order to ensure that there are at least two separate chromosomes to be distributed into the two daughter cells at division, regardless of the growth rate, the frequency of initiation of replication is controlled so that the DNA content of a cell is doubled every generation. That is, if the cell has a doubling time of 20 min then there is one initiation event at each chromosomal origin every 20 min and if the doubling time is 60 min then there is one initiation per origin every 60 min. The result of this is that when cells are growing very fast, they have more than one pair of replication forks per chromosome, that is, overlapping rounds of replication, whereas when the cells are growing very slowly, there is a gap between the end of one round of replication and the start of the next. Therefore initiation of replication (rather than replication itself) is a cell-cycle regulated process. A minor point of regulation also occurs at a point on the chromosome where the two replication forks meet, called the terminus. If one fork reaches the terminus first, it arrests, and the other fork continues, ensuring that both of the replication forks meet at this point (Kuempel *et al.*, 1990).

1.5.1 Initiation of replication

Initiation takes place at a particular time in the cell cycle, once, at every available origin of replication; that is, there is precise timing and synchrony of initiation. Timing of initiation appears to be very closely coordinated to cell growth, such that it takes place at every copy of the origin, at each successive doubling of a fixed unit mass or volume (Donachie, 1968, Helmstetter and Leonard, 1987).

The origin of replication, a stretch of 245 bp called *oriC*, is situated at 84 min on the *E. coli* chromosome. Under normal circumstances, initiation of replication always occurs at *oriC*. In order for this to happen, the large DNA polymerase III holoenzyme must gain access to two separated strands of the DNA, so that it can make a copy of each. This involves 'melting' (opening up) and then unwinding of the DNA duplex at *oriC*. The former is mediated by binding of a protein called DnaA at specific sites in *oriC*. DnaA recognises a 9 bp sequence called a 'DnaA box' at which it binds double stranded DNA. There are four DnaA boxes very close to one another in *oriC*. Binding of 20–40 molecules of DnaA to these boxes in a cooperative manner, causes melting of nearby AT-rich sequences. This allows proteins called DnaB and DnaC to enter the origin, which in turn allows the DNA to be unwound by a helicase in preparation for priming and the action of the DNA polymerase III holoenzyme (Bramhill and Kornberg, 1988). DnaA is the only known protein which is required for initiation and not for later stages in replication. As such it is a very good candidate for the major factor in initiation timing control. This possibility has induced a vast amount of work on the control of DnaA activity and expression of its gene, however it is still not certain whether DnaA provides this ultimate control.

Initiation of replication requires an RNA primer, and some evidence indicates that transcription into the *oriC* region from the promoters of the adjacent *mioC* gene is a factor that influences initiation. Investigation into this gene has shown that it is stringently controlled, so that its expression is in inverse proportion to growth rate (Chiaramello and Zyskind, 1989, Chiaramello and Zyskind, 1990). Also, cell cycle dependent transcription of *mioC* has been detected, such that it is depressed until immediately after the

initiation of replication (Theisen *et al.*, 1993). This expression pattern is thought to be more consistent with a role in stopping reinitiation at the same origin because transcription into the origin causes increased negative supercoiling in the region, making melting of the DNA less likely (Theisen *et al.*, 1993). The same technique was applied to transcription of a nearby gene, *gidA*, which transcribes away from the origin which was found to be depressed until immediately prior to initiation. Transcription away from the origin reduces negative supercoiling in that region and so it is thought that thistemporal control of *gidA* expression would promote the melting of the origin (Theisen *et al.*, 1993). Neither of these observations fit with a role for these genes in priming of initiation, although their transcription could be important auxiliary controls for initiation timing. This picture is complicated by observations that *mioC* transcription is negatively controlled by the DnaA protein and is not actually essential for replication. Transcription, which may enhance initiation of replication, has been detected within the origin itself (Asai *et al.*, 1992, and references therein) and may constitute the initiation primer.

Initiation is synchronised such that all origins initiate at the same time and no one origin is initiated more than once per cell cycle. One aspect of this control (the fidelity or synchronisation of initiation) is the methylation of DNA by the *dam* methyltransferase. This enzyme recognises GATC sequences in DNA and methylates the adenine in this sequence an both strands. All such sites are methylated in mature *E. coli* DNA; however, newly replicated DNA is methylated on the old strand only. Such hemimethylated DNA is not a substrate for initiation of DNA replication (Russell and Zinder, 1987) and so *oriC*, having eleven GATC sites, is refractory to initiation while it remains hemimethylated. Full methylation of new strands of DNA takes only a few minutes, except in the *oriC* region, which remains in the hemimethylated state for 30–40% of the cell cycle, independent of growth rate (Campbell and Kleckner, 1990). Until it becomes fully methylated, *oriC* is associated with the outer membrane (Ogden *et al.*, 1988). It is thought that this series of events, regulated by the methylation state of *oriC*, ensures that each origin can only be a substrate for initiation once per cell cycle; that is,

once the origin is released from the membrane in its methylated and initiation-competent state, other factors required for initiation (e.g. high levels of active DnaA, transcription into the origin) are not available until the next cycle. The importance of this control is shown by the fact that *dam*$^-$ cells have a random timing of initiation, unlinked to cell mass (Bakker and Smith, 1989) and over or underproduction of *dam* methyltransferase produces cells with aberrant copy numbers of *oriC* (Boye and Løbner-Olesen, 1990). This provides a good explanation for synchronous initiation and for the lack of more than one initiation per origin per cycle, but what causes the *oriC* to stay hemimethylated for so long remains unknown. Recently, Herrich *et al.* (1994) have isolated a protein, HobH, which specifically binds the hemimethylated origin. They have sequenced the gene *hobH*, which lies at 92 min on the chromosome, and have shown that cells deleted for the gene exhibit asynchronous initiation (Herrich *et al.*, 1994).

1.5.2 Termination of replication

DNA replication is terminated when the two moving replication forks collide (normally opposite the origin) or alternatively at defined terminus sites which offer a physical block to further replication. These *ter* sites are separated by about 5% of the chromosome and they act in one direction only, such that a fork first passes through the other *ter* site and the DNA between the two, before it is stopped (Hill *et al.*, 1987, deMassy *et al.*, 1987). Thus their role in termination appears to be as 'backstops' which prevent one fork going back towards the origin if the other has been delayed. This backup role is supported by the fact that both sequences can be deleted without serious consequences. Active termination at *ter* sites is mediated by a transacting factor, the Tus protein, which is encoded by a gene (*tus*) very near the terminus region (Hill *et al.*, 1988).

Some form of termination is obviously required before the two copies of the chromosome can be distributed into opposite daughter cells prior to division. As such, correct termination of replication is important for the coordination of replication, partitioning and cell division.

1.6 Chromosome Partitioning

Chromosome partitioning is the process which ensures that, when a cell divides, each daughter cell receives a chromosome. It is often thought of more precisely, as the moving apart of chromosomes into opposite daughter cells before division. If partitioning did not occur, then anucleate (or DNA-less) cells would be produced from dividing cells in which both chromosomes were the same side of the septum. In a growing population of wild-type *E. coli* however, less than 1 in 3333 cells are anucleate (Hiraga *et al.*, 1989), thus the partitioning process is very effective. No visible mitotic apparatus, such as the spindle which carries out chromosome partitioning in eukaryotes, has been found in bacterial cells.

The archetypal model for chromosome partitioning was proposed by Jacob *et al.* (1963) and invoked a relationship between replication and partitioning, and between partitioning and cell growth. It was proposed that replication of the chromosome was triggered by an "initiator" substance attached to the cell surface. When replication had terminated, this left two chromosomes attached to the cell surface and their partitioning was accomplished by localised cell growth between their points of attachment. The predictions made in this model (attachment of the chromosome to the cell surface, a requirement for termination to occur before partitioning, localised cell growth at the centre before division) provided a good basis for experimental investigation of the partitioning process. Thirty years on, a great deal of information has been gathered on the process and its requirements, however the details of the mechanism involved remain elusive.

1.6.1 Relationships between the cell surface and partitioning

Three predictions in the model of Jacob *et al.* (1963) involve associations between the cell surface and chromosome partitioning; [i] that there are surface attachment sites for chromosomes and [ii] that the two replicated chromosomes are separated from one another by movement of these sites and [iii] that the sites move due to localised cell growth in between them.

As mentioned in the previous section on chromosome replication, a physical association between the cell surface and the chromosome has been established (Ogden *et al.*, 1988), in which the hemimethylated origin of replication is sequestered into the outer membrane after initiation of replication. Further work along these lines has identified a membrane fraction which is enriched for fully methylated *oriC* DNA. This fraction has characteristics intermediate between the outer and cytoplasmic membranes, and accounts for over 2/3 of membrane associated *oriC*-binding activity (Chakraborti *et al.*, 1992). These two interactions between the origin of replication and the cell membranes were therefore good candidates for the surface attachments involved in partitioning; however, experiments involving *oriC* plasmids (plasmids which initiate replication from a cloned copy of *oriC*) have placed doubt on this. Membrane-*oriC* complexes have been detected from *oriC* plasmids in the same way as from the chromosome (Katoaka *et al.*, 1991), however *oriC* plasmids are unstably maintained in cells under non-selective conditions (Ogura and Hiraga, 1983) indicating that they are not partitioned. It can be concluded that the membrane association of *oriC* is not sufficient for partitioning; whether it is required for partitioning is not known. *dam*$^-$ strains produce anucleate cells at a low level, however, this is thought to be due to a replication defect rather than a partitioning defect *per se* (Vinella *et al.*, 1992b). This indicates that the membrane sequestration of the hemimethylated origin which is required for initiation synchrony, is not required for correct partitioning. It may be that fully methylated origin DNA is involved in partitioning via an interaction with the membrane and that some other aspect of the chromosome makes its association with the membrane different to that of *oriC* plasmids.

Circumferential zones of adhesion between the cytoplasmic membrane, peptidoglycan and the outer membrane, known as 'periseptal annuli', have been detected upon plasmolysis of *E. coli.* (MacAlister *et al.*, 1983). These structures were proposed to develop at the potential division site of a growing cell, then duplicate and move apart during cell elongation, ending up at cell quarters - the potential division sites of each prospective daughter cell (Cook *et al.*, 1987). As such this organelle was a perfect candidate for the

partitioning attachment site. Subsequent work by the same laboratory showed association of *oriC* DNA with a membrane fraction which had intermediate characteristics between the outer and inner membranes (described above, Chakraborti *et al.*, 1992) and further supported this idea. That is, it seemed to implicate a junction between inner and outer membranes, like that seen in the periseptal annuli, in chromosome attachment to the cell surface. The development of periseptal annuli was studied by observing the positions of plasmolysis bays. These are regions where cytoplasm becomes detached from the cell wall due to exposure of cells to hypertonic solutions, and their edges (where cytoplasm is still attached to the wall) are thought to indicate the presence and position of annuli. Careful analysis of plasmolysis in *E. coli* cells by Mulder and Woldringh (1993) led them to conclude that plasmolysis bays have no relation to the development and positioning of division sites. This result, together with an apparent inability to detect the annular structures in laboratories other than the discoverers', has led to doubts about the involvement of periseptal annuli in partitioning. Indeed, there is evidence that such appositions between outer and inner membranes may be an artefact of electron microscopy preparation techniques (Kellenberger, 1990).

The prediction that cell elongation should be specifically between sites of chromosome attachment in order that they be moved to opposite halves of the cell, has also been investigated. Unfortunately for this proposal, results have shown that the cell wall is synthesised in a dispersed mode rather than in a central zone (Woldringh *et al.*, 1987). This does not rule out localised growth of either of the two cell membranes, but it is difficult to see how such zonal differentiation could be maintained in a semi-fluid membrane.

1.6.2 Relationships between cell division and partitioning

The development of a simple staining technique for DNA which can be used in association with phase contrast microscopy (Hiraga *et al.*, 1989), has led to a great deal of analysis of the distribution of chromosomes in *E. coli*. In a variation of the original method, DNA is condensed into discrete, rounded nucleoids (by inhibiting protein synthesis with chloramphenicol, Zusman *et al.*, 1973) that have been

shown to correspond to single chromosomes in rod-shaped cells (Donachie and Begg, 1989a, Donachie and Begg, 1989b). There is a problem in interpretation however, in that a condensed nucleoid is not the natural state for the chromosome. The condensed nucleoid may not therefore represent the natural distribution of DNA around the cell; however, the non-random positioning of these bodies in wild-type cells at either the centre (in uninucleate cells) or the cell quarters (in binucleate cells) is thought to indicate a relationship between each chromosome and some positioning device like a membrane attachment site. Furthermore, when filamenting division mutants are observed in this way, they have a very regular distribution of nucleoids (e.g. Begg and Donachie, 1991).

The implication of regular nucleoid distribution in filaments is that positioning (and by inference) partitioning of chromosomes is independent of cell division. In direct opposition to this view are experiments by Tétart *et al.* (1992) which indicate that artificial reduction of the level of FtsZ, which delays the onset of septation, similarly delays partitioning. Hence they propose a direct influence of the division apparatus on partitioning. Perhaps more consistent with the first of these two views are observations that actively replicating nucleoids (not condensed by the aforementioned method) can influence the position of division sites (Mulder and Woldringh, 1989).

1.6.3 Relationships between cell shape and partitioning

Observations of wild-type cells has shown that those with two nucleoids almost always have them positioned at the cell quarters (as opposed to together in the middle). Also it has been shown that when protein synthesis is inhibited, initiated rounds of replication can continue to completion, but the resulting pairs of sister chromosomes remain as a single large nucleoid at the centre of the cell. When protein synthesis is allowed to continue, nucleoids start to appear at cell quarters immediately. These observations (Donachie and Begg, 1989b, Hiraga *et al.*, 1990) have been interpreted as meaning that [i] chromosomes are separated very quickly during partitioning and [ii] partitioning requires post-replication protein synthesis. Experiments performed by Donachie and Begg (1989a) investigated the relationship between cell shape and partitioning. They observed

condensed nucleoids in spherical and rod-shaped cells, growing at comparable rates. It was found that the length distribution (diameter in the spherical cells) and the number of nucleoids per cell was virtually identical in the two different populations. Taking account of two facts; [i] that a spherical *E. coli* cell has a volume approximately 6x that of a rod-shaped cell of the same length and [ii] that *E. coli* controls replication initiation in direct relationship to cell volume (or mass), it was concluded that the nucleoids in the spherical cells contained many, fully replicated chromosomes. The amount of DNA in the spherical cells was measured and found to be consistent with this conclusion. Therefore it was proposed that chromosomes can only be partitioned from one another when there is a minimum distance for that partitioning event to occur in (Donachie and Begg, 1989b). Thus partitioning in the spherical cells was delayed until they reached a diameter equivalent to the length at which a rod-shaped cell partitions its two chromosomes. A second set of experiments were conducted where partitioning was stopped for a long period of time while cell growth was allowed to continue. This led to the observation that, when partitioning was allowed to recommence, nucleoids did not quickly move to the quarters of the long filaments but moved away from the central part of the cell by a short distance (Begg and Donachie, 1991). This was taken to indicate that partitioning occurs over a fixed distance. This distance was proposed to be half the minimum cell length at which partitioning occurred in the rod-shaped and spherical cells in the previous experiment (Donachie and Begg, 1989b, Begg and Donachie, 1991).

Taking into account the artificial condensation of the nucleoids in these experiments, the final conclusions should perhaps be, that post-replication protein synthesis and a minimum cell length are required for rapid separation, by a fixed distance, of the points around which chromosomes condense when treated with chloramphenicol. It is possible that these points represent attachment sites of chromosomes to the cell surface, however this has not been proven. Implicit in these conclusions is the quick movement of 'something' from the cell centre to cell quarters, rather than the gradual change associated with growth predicted by Jacob

et al., (1963) and thus it may be reasonable to search for some mitosis-like cytoskeletal apparatus in *E. coli*.

An entirely different view of chromosome partitioning is that the process is an innate property of the nucleoid itself and indeed takes place slowly in concert with cell elongation (Woldringh, 1976). Observations of live cells and fixed but hydrated cells (i.e. the nucleoids are not artificially condensed) showed that the nucleoid grows gradually in length, maintaining a fixed distance between its outer border and the poles of the cells. At some point, the nucleoid splits, resulting in separate nucleoids at the cell quarters without any actual sudden displacement of DNA (Van Helvoort and Woldringh, 1994). It is pointed out that if the positions of the centres of these uncondensed nucleoids were plotted it would give a similar picture to the experiments mentioned previously, that is, it would show a rapid jump from the centre to the two cell quarters. The implication is that nucleoids treated with chloramphenicol, condense around their central position.

A model for 'transcription-translation-mediated' segregation of chromosomes has been proposed from these observations. In this model, the chromosomes move in opposite directions due to the progress of replication and the formation of sub-cellular compartments actively synthesising ribosomal proteins and RNAs. A constant connection with the membrane is maintained through concurrent transcription and translation (the extent of which may be variable). This connection accounts for the final positioning of the nucleoids and therefore no specific chromosome-membrane binding is required (Woldringh and Nanninga, 1985). A similar model has been proposed from observations of L-forms; these wall-less cells can divide, and their nucleoids seem to partition automatically through general interactions with the membrane, which are reflected in the changing shape of the L-forms as the cell cycle progresses (Gumpert, 1993). A consequence of the model of Woldringh and Nanninga (1985) is that chromosomes with multiple replication forks would form nucleoids with complex structures and movement. Observations of rapidly growing cells revealed multi-lobed nucleoids which appeared to move in directions other than along the axis of the cell (Woldringh *et al.*, 1994), however their movement was not found to be dependent

on replication. The lobed nucleoids were seen to cause an increase in cell diameter in their vicinity when they *were* actively replicating and this has been put forward as an explanation for the well documented dependence of diameter on growth rate (Woldringh *et al.*, 1994).

The possibility of a cytokinetic apparatus being involved in chromosome partitioning is not necessarily incompatible with these observations of slow-moving, complex nucleoids. Chromosomes could be guided slowly to either side of the cell by such an apparatus.

1.6.4 Relationships between replication and partitioning

Termination of replication must take place before partitioning can occur (Jones and Donachie, 1973; *1.5.2*) but there are other termination-related processes which must be undergone before the chromosomes can be physically separated.

Mutant strains in which DNA appeared to be stuck in the centre of the cell were initially thought to be partitioning mutants. It is now known that these '*par*' mutations were in genes for proteins with gyrase or topoisomerase activity (reviewed in Hiraga, 1993). The helical structure of DNA results in two newly synthesised daughter chromosomes being catenated at termination. The decatenation of the chromosomes is necessary for their ultimate separation and is carried out by gyrase and topoisomerase proteins (e.g. Adams *et al.*, 1992). The '*par*' mutants were therefore unable to separate their chromosomes before the partitioning apparatus could work.

An aspect of replication which influences the structure of chromosomes at termination is sister chromatid exchange. If an odd number of these recombination events occur, completion of replication will result in a single circular dimer of the two chromosomes. A site-specific recombination event in this circular dimer is then required to produce two unlinked monomers (Løbner-Olesen and Kuempel, 1992). This resolution of dimers is performed by a very active recombination complex of two proteins called XerC and XerD (Blakey *et al.*, 1991). They act at a specific site called *dif*, situated at the terminus of the chromosome (Kuempel *et al.*, 1991). Mutations in either of the Xer proteins or in *dif* cause cells to filament, and as with the gyrase and topoisomerase proteins, their

action is necessary before the partitioning apparatus can successfully carry out its function.

1.6.5 Potential components of the partitioning apparatus

The '*par*' mutants mentioned above were the only proposed partitioning mutants for a long time. Unfortunately their characterisation showed that their gene products were not actually involved in partitioning, but their action was necessary before partitioning could occur. In an attempt to isolate real partitioning mutants, Hiraga and co-workers (1989) devised a screening technique for colonies producing a proportion of anucleate cells. They proposed that cells defective in partitioning may be able to survive, due perhaps to the relative dimensions of chromosomes and cells causing most septa to arise between the two chromosomes anyway. Therefore, the only phenotype of a partitioning defective strain would be the occasional production of anucleate cells. The technique developed to screen mutagenised cells for anucleate cell production worked as follows. A plasmid, with no partitioning system, containing the *E. coli lacZ* gene, was constructed. In a particular host strain, the copy number of the plasmid was low and *lacZ* expression was repressed, both due to chromosomal determinants. If this plasmid were to segregate into an anucleate cell, the negative influence of the chromosome on both copy number and *lacZ* expression would be lifted. In short, colonies containing anucleate cells, would be blue on X-Gal (5-bromo-4chloro-3-indolyl-β-D-galactopyranoside) plates (Hiraga *et al.*, 1989).

Two of these mutants, *mukA* and *mukB*, were shown by microscopic analysis to have the predicted properties. That is, they both produced anucleate cells at a low frequency and showed atypical positioning of condensed chromosomes (Hiraga *et al.*, 1989, Niki *et al.*, 1991). The *mukA* mutation has been shown to be in a previously characterised gene, *tolC*, which codes for an outer membrane protein essential for the production of haemolysin (Hiraga *et al.*, 1989, Niki *et al.*, 1990, Wandersman and Delepelaire, 1990). It is unclear whether Hiraga *et al.* believe this to be involved in partitioning, however as an outer membrane protein it would be a potential attachment site for the chromosome. The second mutation isolated in this way, *mukB*,

had an identical phenotype to *mukA*. The mukB gene has been sequenced and is a novel gene encoding the largest known *E. coli* protein (Niki *et al.*, 1991). Protein structure prediction, purification and electron microscopy has shown that the MukB protein has a novel structure. It has globular amino- and carboxy-terminal domains separated by an alpha-helical rod-shaped region. The amino-terminal domain contains a nucleotide binding site and the carboxy-terminal domain contains three zinc fingers (Niki *et al.*, 1992). The protein is found as a homodimer *in vivo*, which has been shown to form a hinge-like structure whereby it can bend at the middle of the central coiled-rod domain. MukB has been shown to be an ATP/GTP binding protein and has significant homology to eukaryotic cytokinetic proteins such as myosin and dynamin (Niki *et al.*, 1992). All these features of MukB together with its proposed role in partitioning lead to the idea that it is involved in the physical moving apart of chromosomes.

Deletion of mukB is non-lethal at 22°C but shows a partitioning defect at this temperature. The deletion is lethal at higher temperatures and cells form filaments with irregularly spaced chromosomes, suggesting that MukB may be involved in both partitioning and septation. It is not yet known how MukB is involved in partitioning, but it has been suggested that it might drag chromosomes along putative cytoplasmic protein-polymer filaments which either cross the cell or are associated with the inner membrane (Hiraga *et al.*, 1991). Such structures have not yet been found in electron microscopic studies of wild-type cells. Recently however, cytoplasmic bundles of filaments which run longitudinally along cells have been revealed in thin sections of cells containing a high copy number plasmid carrying the *cafA* gene (Okada *et al.*, 1994). This gene is located downstream of the *mre* 'shape-determining' genes (*1.4.1*) and although its deletion has no obvious effect its overexpression produces these 'cytoplasmic axial filaments' and causes cells to produce minicells and anucleate rods (Okada *et al.*, 1994). The role of *cafA in vivo* is not clear.

The discovery of cell-spanning filaments and a putative force generating protein (MukB) makes the existence of a 'mitotic' apparatus in *E. coli* seem more likely. Experiments by Casaregola *et*

al. (1990) were designed to look for proteins in *E. coli* which were related to the eukaryotic force generating protein myosin. They isolated a protein which cross-reacts with a monoclonal antibody raised against yeast heavy chain myosin. This protein has subsequently been found to be identical to the RNase E protein (Casaregola *et al.*, 1992) and whether it has any cytokinetic role is not known.

The characterisation of the contractile ring FtsZ, the formation of which is essential for cell division (*1.3.1*), has provided perhaps the best candidate for a cytokinetic structure in *E. coli* (Lutkenhaus, 1993).

The subject of chromosomal partitioning is fascinating but often difficult to study, being part of a cycle with so many interconnected aspects. Experiments presented in this Thesis were designed to use simple genetics to investigate the nature of partitioning, rather than attempting to characterise any of the structures involved.

Chapter 2
Materials and Methods

2.1 Bacterial and Phage Strains, Plasmids and General Materials

2.1.1 Bacterial strains

Bacterial strains used in this study are listed in Table 2.1.1. Bacteria were either maintained on L-broth plates stored at room temperature, or for longer term storage, in frozen storage buffer at -70°C (see 2.3.2).

Table 2.1.1 Bacterial strains

Strain	Genotype	Source/reference
AJ7	*argG*, *metB*, *rpsL9*; Str^R, *lacY14*, *recA1*, *xyl*, *mal*, λ^R, *mtl*, *rpoB70*; Rif^R	R. Hayward
BL21(DE3)	Kan^R, lysogen of λDE3 [encodes T7 RNA polymerase under *lac*UV_5 promoter control]	Studier and Moffat (1986)
CAG12149	*zbd-601*::Tn*10*; Tet^R	B. Bachmann
C600	*thi1*, *leuB6*, *thr1*, *lacY1*, *hsdR*, *tonA21*, *supE44*	Young and Davis (1983)
D301	*lacY*, *eda*, Δ(*lacIZYA*) U169, *recD1903*::mini-Tet; Tet^R	Laboratory stock
DH5α	ϕ80*dlacZ*ΔM15, Δ(*lacZYA-argF*) U169, *recA1*, *endA1*, *hsdR17* (r_K^-, m_K^+), *supE44*, λ^-, *thi1*, *gyrA96*, *relA1*	Gibco BRL
DH5αF'IQ	As DH5α except *deoR*, F': (*proAB*$^+$, *lacI*^{q} *Z*ΔM15), *zzf*::Tn*5*; Kan^R	Gibco BRL
JC9387	Δ(*gpt-proA*) 62, *argE3*, *his4*, *leu6*, *thr1*, *ara14*, *galK2*, *lacY1*, *xyl5*, *mtl1*, *thi1*, *rpsL31*;Str^R *tsx33*;Tet^R, *recB21*, *recC22*, *sbcB15*, *sbcC201*, *sup*^{0}	D. Leach
FS1585	*recD*, *supE*, *supF*	D. Leach
DB1318	*recD1014*, *recA*::Cm^R, *hsdR2*, *zyg-202*::Tn*10*; Tet^R	D. Leach
FI99	As OV2 except *pbpA99*, *zbe-280*::Tn*10*; Tet^R	This work.
FI139	As OV2 except *holA139*, *zbe-280*::Tn*10*; Tet^R	This work.
FI145	As OV2 except *leuS145*, *zbe-280*::Tn*10*; Tet^R	This work.
FI164	As OV2 except *pbpA164*, *zbe-280*::Tn*10*; Tet^R	This work.

Strain	Genotype	Source/reference
FI205	As OV2 except *holA205*, *zbe-280*::Tn*10*; Tet^R	This work.
FI342	As OV2 except *leuS342*, *zbe-280*::Tn*10*; Tet^R	This work.
FI456	As OV2 except unknown *ts* lethal linked to *zbe-280*::Tn*10*; Tet^R	This work.
FI606	As OV2 except *pbpA606*, *zbe-280*::Tn*10*; Tet^R	This work.
FI625	As OV2 except *pbpA625*, *zbe-280*::Tn*10*; Tet^R	This work.
FI691	As OV2 except probable double mutant linked to *zbe-280*::Tn*10*; Tet^R	This work.
FI709	As OV2 except *leuS709*, *zbe-280*::Tn*10*; Tet^R	This work.
FI866	As OV2 except *leuS866*, *zbe-280*::Tn*10*; Tet^R	This work.
FI915	As OV2 except probable double mutant linked to *zbe-280*::Tn*10*; Tet^R	This work.
FI1159	As OV2 except unknown *ts* lethal linked to *zbe-280*::Tn*10*; Tet^R	This work.
FI1216	As OV2 except *pbpA1216*, *zbe-280*::Tn*10*; Tet^R	This work.
FI1623	As OV2 except unknown *ts* lethal linked to *zbe-280*::Tn*10*; Tet^R	This work.
FI1625	As OV2 except *pbpA1625*, *zbe-280*::Tn*10*; Tet^R	This work.
FI1629	As OV2 except *leuS1629*, *zbe-280*::Tn*10*; Tet^R	This work.
FO499	As OV2 except probable *pbpA* mutant (*pbpA499*), *zbd-601*::Tn*10*; Tet^R	This work
FO544	As OV2 except probable *pbpA* mutant (*pbpA544*), *zbd-601*::Tn*10*; Tet^R	This work.
FO702	As OV2 except *pbpA702*, *zbd-601*::Tn*10*; Tet^R	This work.
FO1022	As OV2 except *pbpA1022*, *zbd-601*::Tn*10*; Tet^R	This work.
FO1165	As OV2 except unknown *fts* mutation linked to *zbd-601*::Tn*10*; Tet^R	This work.
HfrC2	PO68', *relA1*, *leu*, *thi39*::Tn*10;* Tet^R	K.J.Begg
JC411	*arg*, *his*, *lac*, *leu*, *mal*, *met*, *rps*, *thy*, *xyl*, $polA^{ts}$	Laboratory stock
KEN90	ara_{am}, lac_{am}, $galK_{am}$, *galE*, trp_{am}, *ilv*, *his*, *thy*, $ftsZ84^{ts}$	K.J. Begg

Strain	Genotype	Source/reference
KJB24	As W3110 except *rodAsui*$_{am}$, Kan^R	K.J. Begg
LM1	SHA2 transduced to *leu*$^+$ but remaining tet^R [due probably to chromosomal duplication]	This work
LM5	As KJB24 except *recA200*, *recAO*$_c$, [*recA*^{ts}], *srl*::Tn*10*; Tet^R, Kan^R	This work
LM6	As LM5 except *leu*::Tn*9*;Cm^R, Tet^R	This work
N1715	*recA200*, *recAO*$_c$, [*recA*^{ts}], *srl*::Tn*10*; Tet^R	R. Lloyd
ND13	*metB1*, *trpR*, Δ(*trpB–E*)*9*, Δ(*metE*)*113*, *asnA*$^+$, *asnB54*::Tn*5*; Kan^R, *sup*^{0}, *hsdM*$^+$, *hsdR*, *polA21*$_{am}$	Laboratory stock
OV2	*ara*$_{am}$, *lac*$_{am}$, *galK*$_{am}$, *galE*, *trp*$_{am}$, *leu*, *ilv*, *his*, (low) *thy*, *supF*^{ts}	K.J. Begg
SHA2	As W3110 except *leu*::Tn*10*; Tet^R	This work
SHA3	As HfrC2 except *rpoB70*; Rif^R, Tet^R	This work
SHA4	As KJB24 except *leu*::Tn*10*; Tet^R, Kan^R	This work
SHA5	As SHA4 except still *leu*$^+$ [due to chromosomal duplication], Tet^R, Kan^R	This work
SHA6	As SHA5 except Tet^S [due to segregation], Kan^R	This work
SHA7	As SHA5 except *leu*$^-$ [due to segregation], Kan^R	This work
SHA8	As SHA5 from transduction of SHA6 to *leu*::Tn*10*; Tet^R, Kan^R	This work
SHA9	As W3110 except *rpoB70*; Rif^R, *thi39*::Tn*10*; Tet^R	This work
SHA35	As KJB24 except *rpoB70*; Rif^R, *thi39*::Tn*10*; Tet^R, Kan^R	This work
SHA36	As SHA5 except *recA*::Cm^R, Tet^R, Kan^R	This work
SHA41	As SHA5 pSU66 except Tet^S [due to segregation after transformation], Kan^R	This work
SHA42	As SHA5 pSU66 except *leu*$^-$ [due to segregation after transformation], Tet^R, Kan^R	This work
SHA43	As SHA6 except *rpoB70*, [Rif^S due to chromosomal duplication], *thi39*::Tn*10*; Tet^R	This work

Strain	Genotype	Source/reference
SHA55	As SHA5pSU66 except *recA*::Cm^R, Tet^R, Kan^R	This work
SHA58	As KEN90 except *leu*::Tn*10*; Tet^R	This work
SHA59	As TOE1 except *leu*::Tn*10*; Tet^R	This work
SHA60	As W3110 except *recA*::Cm^R	This work
SHA62	As KJB24 except *recA*::Cm^R, Kan^R	This work
SHA74	As W3110 except *recA200*, *recAO*$_c$, [*recA*^{ts}], *srl*::Tn*10*; Tet^R	This work
SHA75	As KJB24 except *zbe-280*::Tn*10*; Tet^R, Kan^R	This work
SHA76	As W3110 except *zbe-280*::Tn*10*; Tet^R	This work
SHA77	As ND13 except *zbe-280*::Tn*10*; Tet^R	This work
SHA87	As C600 pADD44' except *rlpB*::Tet^R (orientation of cassette as in pADD50 - ori1)	This work
SHA88	As SHA87 except (ori2)	This work
SHA89	As W3110 pADD30 except *rlpB*::Tet^R (ori1)	This work
SHA90	As SHA89 except *recA*::Cm^R [from SHA60]	This work
SHA91	As SHA89 except *recA*::Cm^R [from DL654]	This work
SHA92	As C600 except *zbd-601*::Tn*10*; Tet^R	This work
SHA93	As OV2 except unknown *fts* mutation linked to *zbd-601*::Tn*10*; Tet^R [from FO1165]	This work
SHA94	As SHA93 except using C600	This work
SHA95	As SHA93 except using W3110	This work
SHA96	As W3110 pADD30 except *recA*::Cm^R	This work
SHA97	As W3110 except *orfUU*::Kan^R (cassette orientation as in pADD57) [viable due to chromosomal duplication]	This work
SHA98	As W3110 pADD30 except *orfUU*::Kan^R (cassette orientation as in pADD57)	This work
SHA99	As C600 pADD23' except *orfUU*::Kan^R (cassette orientation as in pADD57)	This work
SHA100	As SHA91 except *rlpB*::Tet^R (ori1) is now on plasmid	This work
SHA101	As SHA98 except *recA*::Cm^R [from DL654]	This work
SHA102	As SHA97 except Kan^S [due to segregation]	This work

Strain	Genotype	Source/reference
SHA103	As C600 pADD23' except *orfUU*::Kan^R (cassette orientation as in pADD56)	This work
SK2257	*thyA6*, *rpsl120*, *deoC1*, *zbe-280*::Tn*10*; Tet^R	B. Bachmann
TG1	F'[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*Δ*M15*], *thi1*, *supE*, *hsd*Δ*5*, Δ(*lac-proAB*)	Gibson (1984)
TOE1	*thyA*, *argE*, *his*, *pro*, *thr*, *thi*, *ftsQ*^{ts}	K.J. Begg
W3110	INV: *rrnD-rrnE*, *sup*^{0}	Laboratory stock

2.1.2 Bacteriophage strains

Bacteriophages used in this study are listed in Table 2.1.2. Phage lysates were stored at 4°C as broth suspensions to which a few drops of chloroform had been added to prevent microbial growth. M13 phage lysates were stored without chloroform, M13 being chloroform sensitive.

Table 2.1.2 Bacteriophages

Bacteriophage	Description	Source/reference
P1	Wild-type transducing phage	Laboratory stock
P1*vir*	Virulent transducing phage	Laboratory stock
λwild-type	Wild-type immunity	Laboratory stock
λ*vir*	Virulent	Laboratory stock
λ*dlip5*	Defective transducing phage carrying DNA from the fifteen minute region between the *lip* and *leuS* genes.	Spratt *et al.* (1980)
λ16-2	λ transducing phage carrying DNA from the 2 minute region including the *envA*, *ftsZ*, *ftsA*, *ftsQ*, *murC*, and *ddlB* genes	Lutkenhaus and Donachie (1979)
λ1G6 (168)	λEMBL4 containing stuffer fragment from 14.1 to 14.5 minutes	Kohara *et al.* (1987)
λ15D7 (169)	λEMBL4 containing stuffer fragment from 14.4 to14.8 minutes	Kohara *et al.* (1987)

Bacteriophage	Description	Source/reference
λ3A2 (170)	λEMBL4 containing stuffer fragment from 14.7 to 15.0 minutes	Kohara *et al.* (1987)
λ16A8 (171)	λEMBL4 containing stuffer fragment from 14.9 to15.3 minutes	Kohara *et al.* (1987)
λ3A6 (172)	λEMBL4 containing stuffer fragment from 15.2 to 15.55 minutes	Kohara *et al.* (1987).
M13mp18	M13 based cloning vector	Laboratory stock
M13mp19	M13 based cloning vector	Laboratory stock
M13 no.5	*Hin*dIII/*Bgl*II pADD1 fragment containing *rlpB*, *holA* and N-terminus of *orfUU*, cloned into *Hin*dIII/*Bam*HI restriction of M13 mp18	This work
M13 no.6	*Hin*dIII/*Bgl*II pADD2 fragment containing C-terminus of *orfUU* and *orfU* cloned into *Hin*dIII/*Bam*HI restriction of M13 mp18	This work
M13 no.7	Same pADD2 fragment as for M13 no. 6 cloned into *Hin*dIII/*Bam*HI restriction of M13mp19	This work

2.1.3 Plasmids

Plasmids used and constructed in the course of this study are listed in Table 2.1.3. A guide to the restriction sites used to construct these plasmids is provided in Figure 2.1.1.

Table 2.1.3 Plasmids

Plasmid	Description	Source/reference
pACYC177	p15A replicon, Amp^R, Kan^R	Rose (1988a)
pACYC184	p15A replicon, Cm^R, Tet^R	Rose (1988b)
pADD1	*Mun*I fragment of λ169 containing *rlpB*, *holA*, *orfUU* and *orfU*, cloned into *Eco*RI site of pUC18 [direction of transcription of genes opposite to P*lac*], Amp^R	This work

Plasmid	Description	Source/reference
pADD2	As pADD1 except insert in opposite orientation, Amp^R	This work
pADD5	*Sma*I fragment of λ15D7 containing *ybeA*, *ybeB*, *pbpA*, *rodA* and *rlpA*, cloned into *Sma*I site of pACYC177 [direction of transcription of genes same as interrupted Kan^R gene], Amp^R	This work
pADD6	*Bam*HI omega cassette of pHP45Ω cloned into *Bgl*II site of pADD1 [*orfUU*::Ω], Amp^R, Spc^R, Str^R	This work
pADD7	*Bam*HI omega cassette of pHP45Ω cloned into *Bgl*II site of pADD2 [*orfUU*::Ω], Amp^R, Spc^R, Str^R	This work
pADD8	*Bam*HI omega cassette of pHP45Ω cloned into *Bcl*I site of pADD1 [*rlpB*::Ω], Amp^R, Spc^R, Str^R	This work
pADD9	*Bam*HI omega cassette of pHP45Ω cloned into *Bcl*I site of pADD2 [*rlpB*::Ω], Amp^R, Spc^R, Str^R	This work
pADD10	*Kpn*I/*Hin*dIII fragment of pADD5 containing *rodA* and *rlpA*, cloned into *Kpn*I/*Hin*dIII digested pUC19 [direction of transcription of genes opposite to *lacZ*], Amp^R	This work
pADD11	*Kpn*I/*Hin*dIII fragment of pADD5 containing *rodA* and *rlpA*, cloned into *Kpn*I/*Hin*dIII digested pUC18 [direction of transcription of genes same as *lacZ*], Amp^R	This work
pADD13	*Bam*HI omega cassette of pHP45Ω cloned into *Bam*HI site of pADD14 [*rodA*, *rlpA*::Ω], Amp^R, Spc^R, Str^R	This work
pADD14	pADD11 restricted with *Bam*HI to remove internal fragment of *rlpA* and religated [*rodA*, Δ*rlpA*], Amp^R	This work

Plasmid	Description	Source/reference
pADD17	*Eco*RI/*Sma*I fragment of pADD13 cloned into *Eco*RI/*Sma*I digested pT7-5 [*rodA*, *rlpA*::Ω], Amp^R, Spc^R, Str^R	This work
pADD18	*Bcl*I/*Hin*dIII fragment of pADD11 containing *rlpA* and small C-terminal portion of *rodA*, cloned into *Bam*HI/*Hin*dIII digested pT7-5 [*rlpA*], Amp^R	This work
pADD19	*Bgl*II/*Kpn*I fragment of pADD8 cloned into *Bam*HI/*Kpn*I digested pMAK705 [*rlpB*::Ω, *holA*], Cm^R, Spc^R, Str^R	This work
pADD20	pADD18 restricted with *Bam*HI to remove internal fragment of *rlpA* and then religated [Δ*rlpA*], Amp^R	This work
pADD21	*Bam*HI Kan^R cassette of pUC4-K cloned into the *Bam*HI site of pADD14 [direction of transcription of cassette same as *rlpA*, *rlpA*::Kan^R], Amp^R, Kan^R	This work
pADD23	*Bcl*I/*Hin*dIII fragment of pADD2 containing C-terminus of *rlpB*, *holA*, *orfUU* and *orfU*, cloned into *Bam*HI/*Hin*dIII digested pMAK 705 [direction of transcription of genes opposite to *lacZ*], Cm^R	This work
pADD23'	Complementing plasmid resulting from using pADD56 or pADD57 for chromosomal replacements [theoretically identical to pADD23], Cm^R	This work
pADD24	*Bam*HI fragment of pADD11 containing internal portion of *rlpA*, cloned into *Bam*HI site of pT7-4 [direction of transcription same as Ø10 promoter], Amp^R	This work
pADD26	As pADD24 except insert in opposite orientation, Amp^R	This work

Plasmid	Description	Source/reference
pADD30	*Bam*HI/*Hin*dIII fragment of λ15D7 containing *leuS*, *rlpB*, *holA*, *orfUU*, *orfU*, *ybeA*, *ybeB*, *pbpA* and *rodA,* cloned into *Bam*HI/*Hin*dIII digested pACYC177 [genes transcribed in same direction as interrupted Kan^R gene], Amp^R	This work
pADD31	pADD2 restricted with *Bgl*II/*Bam*HI to remove *orfU* and C-terminus of *orfUU* and then religated [*rlpB*, *holA*], Amp^R	This work
pADD32	pADD5 restricted with *Bam*HI to remove internal portion of *rlpA* and then religated [*ybeA*, *ybeB*, *pbpA*, *rodA*], Amp^R	This work
pADD33	pADD2 restricted with *Bgl*II/*Bcl*I to remove *holA*, N-terminus of *orfUU* and C-terminus of *rlpB* and religated [*orfU*], Amp^R	This work
pADD34	As pADD21 except insert in opposite orientation, Amp^R, Kan^R	This work
pADD35	pADD32 restricted with *Bam*HI/*Bgl*II to remove *rodA* and large C-terminal portion of *pbpA* and then religated [*ybeA*, *ybeB*], Amp^R	This work
pADD36	pADD30 restricted with *Bcl*I/*Bam*HI and then religated, leaving intact *leuS* and N-terminal half of *rlpB*, Amp^R	This work
pADD37	pADD5 restricted with *Bcl*I and then religated leaving intact *rlpA* and small C-terminal portion of *rodA*, Amp^R	This work
pADD38	*Bgl*II Tet^R cassette from pT7-PCR cloned into *Bcl*I restriction of pADD31 [cassette in *rlpB*, with the same direction of transcription as that gene, *rlpB*::Tet^R], Amp^R, Tet^R	
pADD40	*Bgl*II fragment of pADD30 containing C-terminal half of *leuS*, *rlpB*, *holA* and large N-terminal portion of *orfUU*, cloned into *Bam*HI digested pACYC184 [direction of transcription of genes same as interrupted Tet^R gene], Cm^R	This work

Plasmid	Description	Source/reference
pADD41	As pADD40 except insert in opposite orientation, Cm^R	This work
pADD42	*Bgl*II fragment of pADD30 containing *orfU*, *ybeA* and *ybeB* cloned into *Bam*HI digested pACYC184 [direction of transcription of genes same as interrupted Tet^R gene], Cm^R	This work
pADD43	As pADD42 except insert in opposite orientation, Cm^R	This work
pADD44	*Bgl*II fragment of pADD30 containing C-terminal half of *leuS*, *rlpB*, *holA* and large N-terminal portion of *orfUU*, cloned into *Bam*HI digested pMAK705 [direction of transcription of *rlpB* same as *lacZ*], Cm^R	This work
pADD44'	Complementing plasmid resulting from using pADD50 or pADD51 for chromosomal replacements [theoretically identical to pADD44], Cm^R	This work
pADD45	*Bgl*II fragment of pADD30 containing *orfU*, *ybeA* and *ybeB* cloned into *Bam*HI digested pMAK705 [direction of transcription of genes same as *lacZ*], Cm^R	This work
pADD46	pADD40 restricted with *Bst*EII, 'filled-in' and religated to leave only C-terminal half of *leuS*, *rlpB* and small N-terminal portion of *holA*, Cm^R	This work
pADD47	*Bcl*I/*Bgl*II fragment of pADD30 containing *orfU* cloned into *Bam*HI digested pT7-6 [direction of transcription of *orfU* same as Ø10 promoter], Amp^R	This work
pADD49	*Bcl*I/*Bgl*II fragment of pADD30 containing *ybeA*, *ybeB* and small N-terminal portion of *pbpA*, cloned into *Bam*HI digested pT7-6 [direction of transcription of genes opposite to Ø10 promoter], Amp^R	This work

Plasmid	Description	Source/reference
pADD50	*Bgl*II Tet^R cassette from pT7-PCR cloned into pADD44 partially digested with *Bcl*I [cassette in *rlpB*, with the same direction of transcription as that gene, *rlpB*::Tet^R], Cm^R, Tet^R	This work
pADD51	As pADD50 except cassette in opposite orientation, Cm^R, Tet^R	This work
pADD53	*Hin*dIII/*Eco*RI fragment of pADD49 containing *ybeA* and *ybeB* cloned into *Hin*dIII/*Eco*RI digested pT7-5 [direction of transcription of genes same as Ø10 promoter], Amp^R	This work
pADD54	*Bam*HI Kan^R cassette from pUC4-K cloned into partially *Xmn*I restricted, 'filled-in' pADD45 [cassette in *orfU* with the same direction of transcription as that gene, *orfU*::Kan^R], Cm^R, Kan^R	This work
pADD55	*Bam*HI Kan^R cassette from pUC4-K cloned into *Not*I resricted, 'filled-in' pADD45 [direction of transcription of cassette opposite to genes, *ybeA*::Kan^R], Cm^R, Kan^R	This work
pADD56	*Bam*HI Kan^R cassette from pUC4-K cloned into *Bgl*II restricted pADD23 [direction of transcription of cassette same as genes, *orfUU*::Kan^R], Cm^R, Kan^R	This work
pADD57	As pADD56 except cassette in opposite orientation, Cm^R, Kan^R	This work
pADD58	*Sma*I fragment containing pADD18 insert cloned into *Hin*cII restricted pMAK705 [*rlpA*], Cm^R	This work
pADD59	*Hin*dIII restricted PCR product from gap-region, cloned into *Hin*dIII resticted pJW30 in reverse orientation, Amp^R	This work
pADD60	*Hin*dIII restricted PCR product from gap-region, cloned into *Hin*dIII resticted pJW30 in forward orientation, Amp^R	This work

Plasmid	Description	Source/reference
pADD61	*Ava*I/*Bam*HI restricted pADD60, 'filled-in' and religated, Amp^R	This work
pADD62	*Mun*I/*Bam*HI restricted pADD60, 'filled-in' and religated, Amp^R	This work
pADD63	*Eco*RI/*Mun*I restricted pADD60, religated, Amp^R	This work
pADD64	*Hin*dIII/*Bcl*I restricted PCR product from gap-region, cloned into *Hin*dIII/*Bam*HI restricted pJW30, Amp^R	This work
pBR322	pMB1 replicon, Amp^R, Tet^R	Bolivar (1978)
pHP45Ω	Plasmid carrying Ω fragment (Spc^R/Str^R), Amp^R	Prentki & Krisch (1984)
pJW30	pMB1 replicon, terminator detection vector using *gal*K expression, Amp^R	Wright et al. (1992)
pKO1	pMB1 replicon, promoter detection vector, using *galK* expression, Amp^R	McKenny *et al.* (1981)
pMAK705	pSC101 replicon *ts* for replication, Cm^R	Hamilton *et al.* (1989)
pSU66	Plasmid carrying *rodA* gene, Amp^R	S.J. Dewar
pSUZ	*Bgl*II/*Cla*I fragment containing *ftsZ* cloned into *Bam*HI/*Cla*I restricted pBR322, Amp^R	S.J. Dewar
pT7-4	*Col*E1 replicon with polylinker in front of phage T7 Ø10 promoter [Amp^R gene transcribed in the same direction as Ø10 promoter], Amp^R	S. Tabor
pT7-5	Similar to pT7-4 except orientation of polylinker and Amp^R gene reversed, Amp^R	S. Tabor
pT7-6	Similar to pT7-4 except orientation of Amp^R gene reversed, Amp^R	S. Tabor
pT7-PCR	Contains pBR322 Tet^R gene, amplified by PCR, on *Bgl*II fragment, Amp^R, Tet^R	R.W.P. Smith
pUC4-K	Contains Kan^R gene from Tn*903* on *Eco*RI, *Bam*HI, *Sal*I or *Pst*I fragments, pMB1 replicon, Amp^R, Kan^R	Pharmacia Biotech

pUC18	pMB1 replicon, Amp^R	Yanisch-Perron *et al.* (1985)

Plasmid	**Description**	**Source/reference**
pUC19	pMB1 replicon, Amp^R	Yanisch-Perron *et al.* (1985)

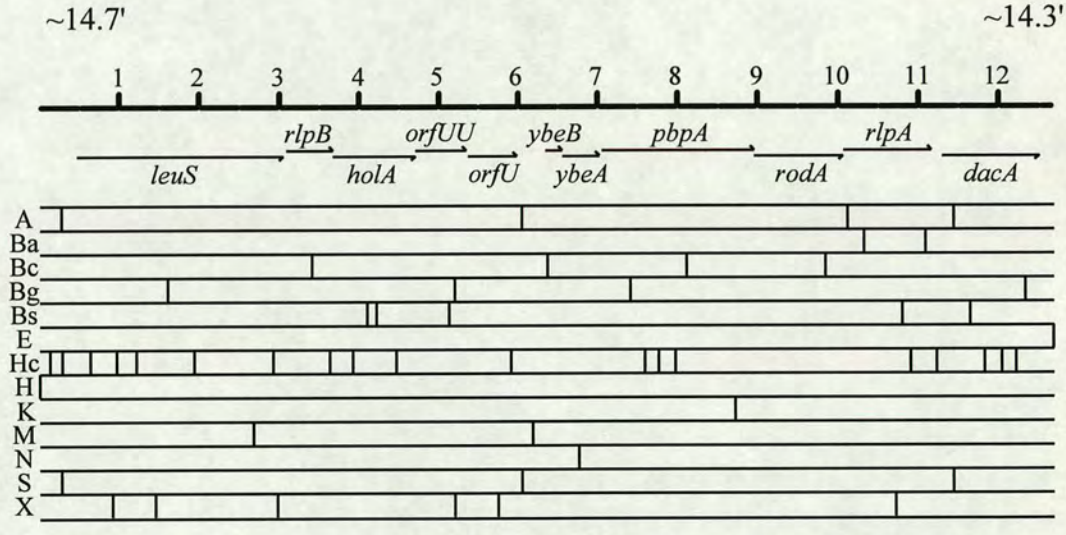


Figure 2.1.1 *Map of the fifteen minute region of the E. coli chromosome showing relevant restriction sites.* The upper line indicates the scale in kilobase pairs (kb). Thin lines with arrow-heads indicate coding regions of genes and their direction of transcription. Short vertical lines indicate the position of restriction sites for the following restriction enzymes: A-*Ava*I; B-*Bam*HI; Bc-*Bcl*I; Bg-*Bgl*II; Bs-*Bst*EII; E-*Eco*RI; Hc-*Hin*cII; H-*Hin*dIII; K-*Kpn*I; M-*Mun*I; N-*Not*I; S-*Sma*I; X-*Xmn*I. Any restriction site mentioned in the plasmid constructions which are not displayed here are in the vector or cassette being used.

2.1.4 Growth media and buffers

Growth media and bacterial/phage buffers are listed in Table 2.1.4. Other commonly used buffers are listed in Table 2.1.5. L-broth (LB) and LB-agar were used routinely for all bacterial manipulations, except where stated. For work with phage λ, media were supplemented with 10 mM $MgSO_4.7H_2O$ and 0.2% maltose (v/v) to maximise expression of the λ receptor protein; for phage P1, 2.5 mM $CaCl_2$ was added.

Table 2.1.4 Growth Media

L-broth (LB)	Difco bacto tryptone	10 g
	Difco bacto yeast extract	5 g
	NaCl	5 g
	pH to 7.2 with NaOH	
	Distilled water to 1 litre	
LB-agar	L-broth + 15 g Difco agar per litre	
LB-top agar	L-broth + 6.5 g Difco agar per litre	
Nutrient broth (NB)	Oxoid No.2 nutrient broth	25 g
	Distilled water to 1 litre	
	It should be noted that NB has insufficient thymine to supplement some thymine-requiring strains; thymine was therefore routinely added at a concentration of 40 μg ml^{-1}	
NB agar	Nutrient Broth + 12.5 g Davis NZ agar	
M9 minimal media	4x M9 salts	100 ml
	Distilled water	300 ml
	$MgSO_4.7H_2O$ (1 M)	0.4 ml
	Carbon source (20% w/v)	5 ml

M9 minimal agar	4x M9 salts	100 ml
	Oxoid water agar	300 ml
	$MgSO_4.7H_2O$ (1 M)	0.4 ml
Oxoid water agar	Oxoid No.3 agar	20 g
	Distilled water to 1 litre	
M9 salts (4x)	Na_2HPO_4	28 g
	KH_2PO_4	12g
	NaCl	2 g
	NH_4Cl	4 g
	Distilled water to 1 litre	
MacConkey agar	Peptone	20 g
	Bile salts No.3	1.5 g
	NaCl	5 g
	Neutral red	0.03 g
	Crystal violet	0.001 g
	Difco agar	15 g
	Distilled water to 1 litre	

Table 2.1.5 Commonly used buffers

TE buffer	10 mM Tris-HCl (pH 8.0)	
	1 mM EDTA (pH 8.0)	
SSC	3 M NaCl	
	0.3 M Sodium citrate	
Phage buffer	Na_2HPO_4	7 g
	KH_2PO_4	3 g
	NaCl	5 g
	$MgSO_4.7H_2O$ (0.1 M)	10 ml
	$CaCl_2$ (0.1 M)	10 ml
	1% gelatin solution	1 ml
	Distilled water to 1 litre	

Bacterial buffer	KH_2PO_4	3 g
	Na_2HPO_4	7 g
	NaCl	4 g
	$MgSO_4.7H_2O$	2 g
	Distilled water to 1 litre	
TAE buffer	*Working solution*:	
	40 mM Tris-acetate	
	2 mM EDTA	
	50x Conc. stock solution:	
	Tris base	242 g
	Glacial acetic acid	57.1 ml
	0.5 M EDTA (pH 8.0)	100 ml
	Distilled water to 1 litre	
TBE buffer	*Working solution*:	
	89 mM Tris-borate	
	89 mM boric acid	
	5x Conc. stock solution:	
	Tris base	54 g
	Boric acid	27.5 g
	0.05 M EDTA (pH 8.0)	20 ml
	Distilled water to 1 litre	

2.1.5 Growth of bacteria

Bacteria were routinely grown as liquid cultures at 37°C (or 30°C for temperature-sensitive strains). Usually fresh overnight cultures that had been inoculated from a single colony were subcultured the following day and grown as required.

Minimal medium supplements. Carbon source (glucose unless indicated otherwise) was added to M9 medium (0.25% w/v) and to MacConkey agar (1% w/v). Amino acid supplements were stored in stock solutions of pure amino acids at a concentration of between 2–10 mg ml^{-1}. Sparingly soluble amino acids, such as tyrosine, were

dissolved in 0.01M NaOH. The final concentration of the amino acids in the media was usually in the order of 20–100 μg ml^{-1}. If rich minimal medium was required, vitamin-free casamino acids (Cαα) were used. The stock concentration of Cαα was 100 mg ml^{-1} and the final concentration in the medium was typically 1–5 mg ml^{-1}. It should be noted that casamino acids lack tryptophan, methionine, uracil and adenine and these should therefore be added to Cαα medium if the strain to be used requires them. Purines and pyrimidines were added to minimal medium when required. Thymine and uracil were stored at a concentration of 2 mg ml^{-1} in water, and their final concentration in minimal medium was usually 20–40 μg ml^{-1}. The only vitamin supplement found necessary in the entire course of this work was thiamine hydrochloride (vitamin B1). This was stored as a 1 mg ml^{-1} solution in water and its final concentration in minimal medium was 2 μg ml^{-1}.

2.1.6 Selection for antibiotic resistance

The routine concentrations for the antibiotics used in this work are shown in Table 2.1.6. All antibiotics were used in both complex and minimal media.

Table 2.1.6 Antibiotic Solutions

Antibiotic	Abbreviation	Solvent	Conc. of stock solution (mg ml^{-1})	Final conc. in media (mg ml^{-1})
Ampicillin	Amp	H_2O	100	50–100
Chloramphenicol	Cm	Ethanol	20	25
Kanamycin sulphate	Kan	H_2O	25	25–50
Rifampicin	Rif	dimethyl-formamide	100	100
Spectinomycin dihydrochloride	Spc	H_2O	50	25–50
Streptomycin sulphate	Str	H_2O	100	20
Tetracycline hydrochloride	Tet	50% Ethanol	10	10

2.2 DNA Techniques

2.2.1 Large-scale plasmid preparation

Isopycnic, caesium chloride gradient ultracentrifugation. A single colony of the plasmid-carrying bacterial strain was inoculated into 5 ml of L-broth with the appropriate selection and incubated overnight at 37°C with vigorous shaking. One millilitre of this culture was then used to inoculate 500 ml of L-broth, with similar selection, in a 2 litre flask, which was then incubated at 37°C overnight, again with vigorous agitation. The culture was chilled on ice, transferred to two 250 ml centrifuge bottles and centrifuged in a Sorvall Superspeed centrifuge at 5000 r.p.m. for 10 min at 4°C. The cell pellets were then each washed in 100 ml of TE buffer, pooled to give a total volume of 200 ml, and recentrifuged as above. The resultant cell pellet was resuspended in 5 ml of a solution containing 50 mM Tris-HCl (pH 8.0) and 25% sucrose and transferred to a 50 ml centrifuge tube. One millilitre of lysozyme (20 mg ml^{-1}) was added, the solution mixed thoroughly, and incubated on ice for 10 min. One millilitre of 0.5 M EDTA (pH 8.0) and 0.8 ml of RNase A solution (10 mg ml^{-1}) were added and incubation continued for a further 10 min on ice. Finally, 5 ml of a lysis solution containing 100 mM Tris-HCl (pH 8.0), 125 mM EDTA, and 0.2% (w/v) Triton X-100 was added, the solution mixed gently but thoroughly by inversion and incubated on ice for another 10 min. The resulting suspension was then centrifuged using a Sorvall SS34 rotor at 15000 r.p.m. for 20 min at 4°C. The plasmid-containing supernatant was then subjected to isopycnic gradient ultracentrifugation to separate plasmid and chromosomal DNA.

CsCl (17.1 g) was dissolved in the supernatant in a 25 ml measuring cylinder. Ethidium bromide (342 µl) solution (10 mg ml^{-1}) was added and the total volume made up to 23 ml with TE. This gave a CsCl density of 1.55 g ml^{-1} and an ethidium bromide concentration of 200 µg ml^{-1}. The solution was then transferred to two 11.5 ml Sorvall Ti50 crimp-seal centrifuge tubes, balanced to within 0.05 g and then centrifuged in a Sorvall 50-B or 55-B ultracentrifuge at 38000 r.p.m. for 60 h at 20°C in a Ti50 rotor. At the end of the run the tubes were carefully removed from the rotor and the DNA bands could be visualised using a UV lamp. The lower (denser) plasmid

bands were removed from the tubes using a syringe fitted with a wide-bore needle. The two samples were then pooled and the ethidium bromide extracted at least five times with isobutanol (isobutanol over CsCl-saturated TE). The sample was then dialysed against several changes of TE (1:2500) at 4°C over a period of 48 h to remove the CsCl. The plasmid DNA was then recovered from solution by precipitation (*2.2.2*).

Large-scale alkaline lysis method. This method, based on that described by Ausubel *et al.* (1994), was preferred when large amounts of plasmid DNA were required quickly without the need for the extreme purity of the CsCl method.

A single colony of the plasmid-carrying bacterial strain was inoculated into 250 ml of L-broth with the appropriate selection and incubated overnight at 37°C with vigorous shaking. The cells were collected in a 250 ml bottle by 10 min centrifugation at 5000 r.p.m. in a Sorvall Superspeed centrifuge, using a GSA rotor at 4°C and the supernatant discarded. The cell pellet was resuspended in 20 ml of a 25 mM Tris-HCl, 50 mM EDTA (pH 8.0) solution and then 40 ml of a solution containing 0.2 M NaOH and 1% SDS was added, mixed and then left on ice for 10 min. A heavy flocculent was formed by mixing this with 30 ml of cold 5 M potassium acetate solution and leaving it on ice for 5 min. The flocculent was removed by centrifugation as above for 15 min at 10000 r.p.m. and the supernatant was transferred to a fresh 250 ml centrifuge bottle, 0.6 volumes of isopropanol added, mixed and left at room temperature for at least 5 min. After centrifugation for 15 min at 10000 r.p.m., as above, the supernatant was discarded and the pellet dissolved in 5 ml of TE buffer. To this 2.4 ml of 8 M ammonium acetate solution was added, mixed and left on ice for 20 min. The precipitate was removed by centrifugation for 15 min at 10000 r.p.m. as above and the supernatant was transferred to a 30 ml glass Corex tube. To this 15 ml of ice-cold ethanol was added, mixed and left at room temperature for at least 5 min. The DNA precipitate was collected by centrifugation for 15 min at 10000 r.p.m. in a Sorvall Superspeed centrifuge, using an SS34 rotor at 4°C. The supernatant was removed, the pellet air-dried and then resuspended in 750 µl of TE

buffer. To this an equal volume of Tris-saturated phenol (pH 8.0) was added. The mixture was vortexed, centrifuged for 2 min in a microfuge and the upper (aqueous) phase removed. This was repeated twice and then further extracted twice using chloroform. The resulting DNA solution was then ready for precipitation.

2.2.2 DNA precipitation

DNA was precipitated from aqueous solution by;

(i) Adding 1/10 volume of 3 M sodium acetate (pH 5.5) and 3 volumes of absolute ethanol, mixing thoroughly and leaving on ice for a minimum of 10 min. This was then centrifuged in a microfuge at 15000 r.p.m. for at least 15 min. The supernatant was discarded, and the pellet washed in 70% ethanol by vortexing. This was recentrifuged as above for 10 min, the supernatant again discarded, and the pellet was dried under vacuum. The dried DNA pellet could then be resuspended in a suitable volume of TE buffer (with added RNase to a final concentration of 20 $\mu g\ ml^{-1}$ if required).

(ii) Instead of 3 volumes of absolute ethanol, 1 volume of isopropanol could be used. This had the advantage of keeping the total volume smaller and was therefore the preferred method. After isopropanol precipitation and centrifugation, the pellet was washed with 70% ethanol as above.

2.2.3 Determination of DNA concentrations

DNA concentrations were determined by measuring the absorption of diluted solutions at 260 nm. For double-stranded DNA, an OD_{260} value of 1.0 represents a DNA concentration of 50 $\mu g\ ml^{-1}$, and for single-stranded DNA a similar value represents a DNA concentration of 40 $\mu g\ ml^{-1}$.

DNA purity can be determined by measuring absorption at 260 and 280 nm. Protein-free double-stranded DNA should give a 260/280 ratio close to 1.8, and single-stranded DNA should give a ratio nearer 2.0.

2.2.4 Small-scale plasmid preparation

Routine preparations of plasmid DNA were performed using a modification of the alkaline lysis method of Birnboim and Doly (1979). Five milliltres of L-broth (plus suitable antibiotic selection) was inoculated with a single colony of the plasmid-bearing strain, and incubated overnight with continuous shaking at the appropriate temperature (typically 37°C). The culture was then centrifuged at 4000 r.p.m. for 10 min in a bench-top centrifuge. The supernatant was discarded, and the bacterial pellet resuspended in 0.1 ml of buffer containing 1% glucose, 10 mM EDTA, and 25 mM Tris-HCl (pH 8.0). To this cell suspension 0.2 ml of 0.2 M NaOH/1% SDS solution was added, mixed by gentle inversion of the tube and incubated on ice for 5 min. To this 150 µl of 3 M sodium acetate (pH 5.5) was added, the solution mixed by vortexing, and left on ice for a further 5 min. The mixture was then centrifuged in a microfuge for 10 min in order to pellet the precipitated chromosomal DNA and insoluble cellular debris. The resulting supernatant (~0.5 ml) was transferred to a fresh Eppendorf tube and 0.5 ml of phenol-chloroform-isoamyl alcohol (Tris-saturated phenol (pH 8.0), chloroform and isoamyl alcohol in a 25:24:1 ratio) was added, mixed by vortexing and centrifuged in a microfuge for 2 min. The upper (aqueous) phase was transferred to a fresh tube and the plasmid DNA could then be recovered from solution by ethanol or isopropanol precipitation. It was unnecessary to add extra salt for precipitation to occur. Typically the final pellet of nucleic acid was resuspended in TE buffer containing RNase A (20 µg ml^{-1}). It was usually observed that 5 ml of overnight culture yielded approximately 3–5 µg of plasmid DNA.

2.2.5 Restriction of DNA

Endonuclease cutting of DNA was typically performed in volumes of between 20 and 100 µl. These contained the requisite amount of DNA (usually 1–10 µg) and the appropriate Boehringer Mannheim restriction buffer at 1x concentration. The restriction enzyme was usually present in a two- to fivefold excess, i.e. 2–5 units per microgram of DNA. The digests were made up to their final volume using distilled water. The complete restriction digests were incubated at the recommended temperature (usually 37°C) for 1–3 h. The

products of the reaction were either directly analysed by agarose gel electrophoresis, or phenol extracted, precipitated and dissolved in a suitable volume of TE buffer for further manipulations.

Partial digestion of DNA. For partial digestion of DNA, ten two-fold serial dilutions of restriction enzyme were added to fixed amounts of DNA, with 0.5 units of enzyme per microgram of DNA representing the highest enzyme:DNA ratio. The digests were incubated at the appropriate temperature for 30 min and terminated by addition of tracking dye (see *2.2.9*). The products of the reactions could then be analysed by agarose gel electrophoresis.

2.2.6 Ligation of DNA

Ligations of DNA were typically performed in a final volume of 10 µl. These contained between 0.5–1 µg total DNA with insert DNA in a 2- to 20-fold molar excess over the vector DNA, 1x Boehringer Mannheim ligation buffer and T4 DNA ligase. 0.2 units of ligase was used for the ligation of cohesive DNA termini and 1 unit of the enzyme for the ligation of blunt-ended molecules. The reactions were incubated for at least 12 h at 16°C. Between 5 and 10 µl of the reaction mixture was then used to transform competent cells of an appropriate strain of *E. coli.*

2.2.7 'Filling in' of recessed 3' termini

Klenow enzyme was used to fill-in the recessed 3' termini generated by various restriction enzymes to give blunt-ended DNA molecules. Reactions were performed in a final volume of 20 µl containing 1 µg DNA, 1x buffer (Boehringer Mannheim restriction buffers were found to work well), all four dNTPs each at a concentration of 20 µM and 2 units of Klenow enzyme. The reactions were incubated at 16°C for 45 min. The reactions were stopped and the unincorporated nucleotides removed by increasing the reaction volume to 200 µl with TE, phenol extracting and precipitating the DNA.

2.2.8 Amplification of DNA using the Polymerase-Chain-Reaction

Specific regions of DNA were amplified using the Polymerase-Chain-Reaction (PCR). Either chromosomal or plasmid DNA could be used

as a template and specific oligonucleotide primers were obtained commercially (Oswel DNA Service, University of Edinburgh). A typical reaction mixture for a plasmid template was as follows (chromosomal template DNA was used at 10x the concentration of plasmid DNA):

10x Thesit Buffer III	5 µl
dNTP mix (1 mM for dA, C, G, and TTP)	5 µl
oligonucleotide primer 1 (50 µM)	1 µl
oligonucleotide primer 2 (50 µM)	1 µl
template DNA (10 ng μl^{-1})	1 µl
Promega Taq polymerase (5 units μl^{-1})	1 µl
distilled water	36 µl

Thirty microlitres of mineral oil was layered on top of the reaction mixture and then reactions were carried out in a Hybaid™ Thermal Reactor programmed according to the length of the desired product and the approximate melting temperature of the primer/template duplex.

Thesit Buffer III (10x)	300 mM Tricine pH 8.4
(Ponce and Micol, 1992)	20 mM MgCl2
	50 mM β-mercaptoethanol
	0.1% Gelatin
	1% Thesit

2.2.9 Agarose gel electrophoresis

Agarose gel electrophoretic analysis of DNA was always performed using TAE buffer. The gels were made-up by melting the appropriate amount of agarose (usually between 0.8 and 1.5%, w/v) in 1x TAE buffer over a bunsen-burner. Gels were cast in 11 x 14 cm Pharmacia gel trays and, once set, the DNA samples containing 1x tracking dye (6x tracking dye is 0.25% bromophenol blue, 0.25% xylene cyanol and 40% (w/v) sucrose in H_20) were loaded into the wells at one end of the tray. Pharmacia gel electrophoresis tanks were used with the gels only just immersed in 1x TAE buffer. Electrophoresis was usually performed overnight at a constant current of 25 mA. After completion of electrophoresis, gels were stained in water containing 2

μg ml^{-1} ethidium bromide for about 1 h with constant shaking, and subsequently destained in fresh water for 30 min. The gels could then be photographed using Polaroid film and UV transillumination.

2.2.10 Isolation of DNA from agarose gel slices

To isolate DNA from agarose gels the Geneclean II® method was employed. Geneclean II® is a product of Bio101 and utilises a silica matrix (glassmilk®) which binds DNA in high-salt but not in low-salt solutions. The appropriate DNA band is located in an ethidium bromide-stained gel under UV transillumination and cut out using a clean razor blade in as small a volume of agarose as possible. Gel slices were transferred to Eppendorf tubes, the weight of the slice determined and 3 volumes (300 μl to 100 mg) of saturated sodium iodide solution added. These were incubated at 50°C until the gel slice had dissolved. Five microlitres of glassmilk was added, the suspension mixed well and put on ice for 5 min. Tubes were briefly spun in a microfuge and pellets washed three times in 300 μl of Geneclean New-wash® solution (an alcohol-based washing solution supplied with the kit), centrifuging and resuspending the pellets each time. After the final wash, all traces of the wash solution were removed using a Pasteur pipette and the pellets were then resuspended in 5 μl TE buffer. These were incubated at 50°C for 2–3 min, centrifuged for 30 seconds and the DNA-containing supernatant transferred to a fresh tube. A further 5 μl of TE buffer was added to the glassmilk pellets and the procedure was repeated to give a final DNA-containing solution with a volume of 10 μl. This DNA solution was then used directly for further manipulations or stored at 4°C.

2.2.11 Labelling DNA fragments by random-priming

Preparation of probe DNA. The DNA to be labelled was gel-purified as in *2.2.10*; this was done twice for plasmid fragments, to avoid carry-over of other fragments on the gel, whereas once was sufficient for PCR products.

Incorporation of radioactive label. The DNA solution was made up to 33 μl with distilled water, placed in a boiling water-bath for 3 min and then equilibrated at 37°C for 10 min. The random-priming

reaction was set up in an Eppendorf tube as follows and then incubated overnight at room temperature:

oligo-labelling buffer (OLB)	10 µl
bovine serum albumin (10 mg ml^{-1})	2 µl
[α-^{32}P] dCTP (10 mCi ml^{-1})	5 µl
probe DNA	33 µl
DNA polymerase I Klenow fragment	2 units

OLB consists of a 1:2.5:1.5 mixture of solutions A:B:C as follows:

Solution O	1.25 M Tris-HCl 0.125 M $MgCl_2$ (pH 8.0)	
Solution A	Solution O	1 ml
	β-mercaptoethanol	18 µl
	dATP (0.1 M)	5 µl
	dCTP (0.1 M)	5 µl
	dGTP (0.1 M)	5 µl
	dTTP (0.1 M)	5 µl
Solution B	2 M HEPES buffer (pH 6.6)	
Solution C	Random hexadeoxyribonucleotides at 90 OD units ml^{-1} in TE (Pharmacia Biotech)	

'Cleaning-up' of labelling reaction. Random-primed DNA was separated from unincorporated nucleotides using ELUTIP-d™ columns (Schleicher & Schuell Dassel Germany) according to the manufacturer's instructions. The final eluant from the column was then ready to be used for Southern hybridisation.

2.2.12 Preparation of chromosomal DNA

Large-scale method. One millilitre of a fresh overnight culture of the appropriate strain was used to inoculate 100 ml of L-broth, which was incubated at a suitable temperature overnight with constant agitation. The culture was then chilled on ice and transferred to a

250 ml centrifuge bottle and centrifuged at 5000 r.p.m. in a Sorvall Superspeed centrifuge using a GSA rotor for 15 min at 4°C. The supernatant was removed and the bacterial pellet was resuspended in 20 ml of STE, (TE buffer plus 10 mM sodium chloride). One millilitre of 10% SDS solution and 1 ml of proteinase K solution (4 mg ml^{-1}) were added, mixed gently and incubated at 50°C for 6 h without shaking. To this solution an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, the suspension mixed gently by inversion and allowed to stand at room temperature for 10 min. The mixture was centrifuged in a benchtop centrifuge for 15 min to separate the aqueous and phenolic phases. The upper (aqueous) phase was then carefully removed avoiding the protein interface. The nucleic acids in this phase were precipitated by adjusting the sample to 0.2 M sodium acetate (pH 5.5) and gently layering 2 volumes of ice-cold ethanol on top. The DNA was collected at the aqueous-ethanol interface by spooling it out with a glass rod. The spooled DNA was washed in 70% ethanol, dried briefly in air and dissolved overnight in 10 ml of TE at room temperature. To this solution 0.1 ml of RNase A (10 mg ml^{-1}) was added and the mixture incubated at 37°C for 1 h. Five hundred microlitres of 10% SDS solution and 250 µl of proteinase K solution (4 mg ml^{-1}) were now added and the mixture incubated at 50°C for 1 h. The sample was extracted with phenol-chloroform-isoamyl alcohol and the DNA precipitated by spooling as above. After washing in 70% ethanol the DNA was air-dried and solubilized in 1 ml of TE. This solubilization took between 1 and 3 days. The yield of DNA was determined by UV spectrophotometry (*2.2.3*); from 100 ml of culture about 500 µg of chromosomal DNA was typically obtained.

Small-scale method. The method of Redfield and Campbell (1987) was found to be a reliable protocol for obtaining chromosomal DNA quickly. 1.5 ml of a fresh overnight culture of the appropriate bacterial strain was put into an Eppendorf tube and centrifuged for 2 min in a microfuge. The supernatant was discarded and the pellet gently resuspended in a solution containing 40 mM Tris-HCl (pH 8.0), 20 mM EDTA and 10 mg ml^{-1} lysozyme. This was incubated at 37°C for 30 min. To this 100 µl of a solution containing 5% SDS and RNase

A (100 μg ml^{-1}) was added, mixed by gentle inversion and incubated at 37°C for 5 min. The resulting lysate was extracted with Tris-saturated phenol twice and with chloroform once, taking care to always mix the solutions very gently (usually on a rotating wheel for 5 min). The upper (aqueous) phase was removed using a Gilson P1000 with a cut-off pipette tip to avoid shearing and then the DNA was precipitated by adding 15 μl of 5M NaCl and 1 ml of ethanol, gently inverting and leaving for at least 30 min. Routinely the pellet was resuspended in 50 μl of distilled H_2O and then half of this used for a restriction digest.

2.2.13 Preparation of bacteriophage M13 DNA

In all preparations of M13 DNA the *E. coli* strain TG1 was used. A fresh overnight culture of TG1 was used to set up a culture infected with bacteriophage M13. Fifty microlitres of the overnight culture was used to inoculate 2 ml of L-broth. To this either 100 μl of an M13 phage suspension, (about 1/10 of a single plaque), or an entire M13 plaque from an agar plate was added. This culture was incubated at 37°C with vigorous shaking for about 5 h. One-and-a-half millilitres of this culture was transferred to an Eppendorf tube and centrifuged in a microfuge for 5 min. The resulting bacterial pellet could be used to prepare the double-stranded replicative form of M13 DNA, and the supernatant used to prepare single-stranded M13 DNA (or as a fresh bacteriophage suspension).

Preparation of double-stranded M13 DNA. The bacterial pellet was washed once in bacterial buffer and the double-stranded DNA isolated in essentially the same way as was described for the small-scale isolation of plasmid DNA (*2.2.4*).

Preparation of single-stranded M13 DNA. Between 1.2–1.3 ml of the bacteriophage suspension was transferred to an Eppendorf tube and 200 μl of a solution containing 20% (w/v) polyethylene glycol (PEG8000) in 2.5 M sodium chloride was added, the solution mixed thoroughly and allowed to stand at room temperature for 15 min. The precipitated bacteriophage particles were recovered by centrifugation in a microfuge for 5 min and the supernatant was removed carefully

leaving the pellet as dry as possible. The bacteriophage pellet was resuspended in 100 µl TE with vigorous vortexing. Fifty microlitres of Tris-saturated phenol was added and the suspension mixed thoroughly by vortexing for 1 min. This was then centrifuged for 2 min and the upper (aqueous) phase carefully removed and placed in a fresh tube. The volume of the sample was adjusted to 0.5 ml and the single-stranded DNA purified by successive phenol–chloroform and chloroform extractions and then recovered by ethanol precipitation (*2.2.2*). Using this method the yield of single-stranded DNA tended to be approximately 5–10 µg DNA per millilitre of infected culture and was of sufficient quality for DNA sequencing.

2.2.14 Southern blotting procedures

Transfer of DNA from agarose gels to nylon membranes. DNA was digested with the desired restriction enzymes, electrophoresed through agarose, stained and photographed as described (*2.2.9*). The DNA fragments were depurinated by soaking the gel in 2 volumes of 0.25 M HCl with gentle agitation for 15 min. This was repeated once with fresh 0.25 M HCl. The gel was then rinsed with distilled water and the DNA denatured by soaking in 2 volumes of 0.5 M NaOH/1.5 M NaCl with gentle agitation for 15 min. This was repeated once with fresh denaturing solution. The gel was then soaked in a neutralising solution containing 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2) and 1 mM EDTA for at least 30 min. The gel was finally rinsed in distilled H_2O.

A Boehringer Mannheim positively charged nylon membrane (*sic*) and eight sheets of blotting paper were cut to the same size as the gel. Two sheets of blotting paper (saturated with 20x SSC) were placed on a clean glass plate in a plastic tray. The tray contained 20x SSC, and the pieces of paper were arranged so that they hung over the edge of the plate to act as wicks. Three more saturated sheets of blotting paper and then the pretreated gel, were placed on top of this. The nylon membrane was placed on top of the gel, taking care to exclude any air bubbles; this was followed by a further three sheets of saturated blotting paper. On top of this a 2–4 cm thick wad of dry paper towels was placed, followed by another glass plate. The whole structure was weighted to provide even pressure and maintain good contact between the gel, nylon, and paper towels and transfer was

allowed to continue overnight. The dried-up gel was routinely stained with ethidium bromide to check that transfer was successful, while the nylon membrane was rinsed in 20x SSC, blotted dry, and UV irradiated to cross-link DNA to the filter. This was done in a Stratagene UV Stratalinker™ on the "autocrosslink" setting (1200 μjoules, 254 nm).

Hybridisation of labelled probe to nylon membranes. All steps were carried out using Techne hybridisation bottles in a Techne 'Hybridiser HB-1D' oven. An adaptation of the method of Church and Gilbert (1984) was used. The nylon membrane was pre-hybridized in 20 ml Hybridisation Buffer for at least 2 h at 65°C: this was to prevent non-specific hybridisation of the probe to the membrane. The labelled probe (*2.2.11*) was denatured by placing in a boiling water-bath for 5 min. This was added to 10 ml of fresh hybridisation buffer and incubated overnight with the membrane at 65°C to allow hybridisation. The temperature could be dropped depending on the desired stringency conditions. The probe solution was decanted off and stored at –20°C for possible re-use, and the filter was washed with 10 ml Wash Solution, for 10 min, at 42°C. This was repeated twice. The membrane was checked with a radiation monitor and if the background was still high, it was washed for 10 min with 10 ml Wash Solution at 65°C. The membrane was placed in an air-tight polythene bag, put into an autoradiography cassette with Cronex® X-ray film and stored at -70°C. The time for which the film was exposed to the filter was dependent on the strength of the signal.

Hybridisation Buffer	1 mM EDTA (pH 8.0) 0.5 M sodium phosphate buffer (pH 7.2) 7% (v/v) SDS
Wash Solution	1 mM EDTA (pH8.0) 40 mM sodium phosphate buffer (pH 7.2) 5% (v/v) SDS

2.2.15 DNA sequencing techniques

DNA sequencing was performed using the Pharmacia T7 Sequencing Kit. The kit is based on the chain-terminating dideoxynucleotide sequencing method developed by Sanger *et al.* (1977) however T7 DNA polymerase is used for the elongation reactions instead of the Klenow fragment of *E. coli* DNA polymerase I.

Annealing of primer to single-stranded template. The DNA templates used in these sequencing reactions were all single-stranded M13 DNAs and were purified as mentioned previously. The concentration of the template was adjusted to 1 μg μl^{-1} in TE. In some cases the Universal Primer supplied in the kit was used however in most cases a specific oligonucleotide primer was obtained commercially (Oswel DNA Service, University of Edinburgh) and its concentration adjusted to that of the Universal Primer (0.80 μM). The following was added to an Eppendorf tube on ice:

template DNA (1 μg μl^{-1})	2 μl
primer (0.80 μM)	2 μl
Pharmacia annealing buffer	2 μl
water	8 μl
Total	14 μl

The contents of the tube were mixed well and incubated at 60°C for 10 min. The tube was then left at room temperature for at least 10 min; if the rest of the sequencing reaction was to be performed at a later time then the tube could be stored at -20°C until required.

Annealing of primer to double-stranded template. Double-stranded plasmid DNA prepared by the CsCl large scale method was used as template DNA. The template DNA was adjusted to about 0.25 μg μl^{-1} and then denatured by adding 8 μl of this to 2 μl of 2 M NaOH in an Eppendorf tube. The tube was vortexed gently and then centrifuged briefly in a microfuge. It was then incubated at room temperature for 10 min. The denatured template was then precipitated; 3 μl of 3 M sodium acetate (pH 5.5), 7 μl of distilled H_2O and 60 μl of ethanol were added, mixed and placed on dry-ice for 15 min and the

precipitate was collected by centrifugation and washed as described before (*2.2.2*). The DNA was resuspended in 10 µl of distilled H_2O. For annealing, this was added to 2 µl of annealing buffer and 2 µl of primer solution and incubated at 37°C for 20 min. The tube was then left at room temperature for at least 10 min; if the rest of the sequencing reaction was to be performed at a later time then the tube could be stored at –20°C until required.

Sequencing reaction. For each template to be sequenced, four Eppendorf tubes or wells of a microtitre plate were labelled 'A', 'C', 'G' and 'T' respectively and 2.5 µl of the corresponding dideoxynucleotide mix added to each tube or well. To the tube containing the annealed template and primer the labelling mix, (dCTP, dGTP and dTTP in solution), T7 DNA polymerase and [α-^{35}S] dATPαS were added as follows:

annealed template and primer	14 µl
Pharmacia labelling mix	3 µl
[α-^{35}S] dATPαS (10 µCi µl^{-1})	1 µl
diluted T7 DNA polymerase (1.5 units µl^{-1})	2 µl

This labelling reaction was incubated at room temperature for 5 min. While this was proceeding the previously dispensed sequencing mixes were incubated at 37°C for one minute in a water bath. After the 5 min incubation of the labelling reaction, 4.5 µl was added to each of the pre-warmed sequencing mixes and returned to the water bath for a further 5 min to allow chain-termination to occur. Finally, 5 µl of Pharmacia Stop Solution was added to each reaction, which could then be stored at –20°C until required for electrophoresis. When the samples were needed for loading onto the sequencing gel they were heated to 80°C for 2 min to denature the DNA. Immediately after this incubation 1.5 to 2.5 µl of each sample was loaded onto the gel.

DNA sequencing gel electrophoresis. DNA sequencing was performed using a 30 x 40 cm BRL sequencing apparatus. The glass sequencing gel plates were thoroughly cleaned with ethanol and acetone,

assembled using 0.2 mm spacers and taped together carefully to minimise the possibility of leakage.

The gel was prepared by adding together the following:

urea	43 g
water	35 ml
10x TBE	10 ml
stock acrylamide	15 ml

This was allowed to dissolve with the aid of magnetic stirring. Once dissolved, 1 ml of a 10% (w/v) ammonium persulphate solution was added followed by 35 µl of TEMED. This was stirred slowly for a few seconds and was then poured between the sequencing plates. The flat edge of a 60-well shark-tooth comb was pushed between the plates to layer the top of the gel. Cling-film was wrapped round the exposed areas of the gel and each edge of the gel was clamped with bulldog clips. The gel was then set aside for at least 10 min to allow polymerisation. Once set, the bulldog clips, tape and comb were removed and the top of the gel washed with distilled water. The shark-tooth comb was then replaced with the points downwards just touching the surface of the gel. The gel was clamped into the sequencing apparatus and 1x TBE solution poured into the top and bottom reservoirs. The gel was then pre-electrophoresed at 66 W (~1500 V) for 1 h. After this the sample wells were quickly washed with 1x TBE solution and gel was ready to be loaded with the sequencing reactions. The samples were loaded in the order A, C, G and T immediately after denaturing the DNA (see above). The gel was then electrophoresed at 66 W at least until the bromophenol blue dye-front ran off the end of the gel, the time being varied according to the distance from the primer which was to be read. Once electrophoresis was complete the glass plates were removed from the apparatus and the top plate very carefully lifted off. The bottom plate (with the gel attached) was placed in a fixing bath containing 10% (v/v) methanol and 10% (v/v) acetic acid for 20 min. The plate and gel were then removed and two damp sheets of blotting paper placed on top of the gel followed by two sheets of dry blotting paper. Even

pressure was applied and the papers were peeled off the glass plate taking the gel with them. The gel and paper sandwich was then dried in a vacuum gel-drier for 1 h at 80°C. When dry, the gel was placed in an autoradiography cassette with Cronex® X-ray film and allowed to develop at room temperature. In most cases a good signal was achieved after 24–48 h.

Stock acrylamide

37 g acrylamide, 1 g NN' methylene bis-acrylamide, made up to 100 ml with distilled water, filtered and stored at 4°C.

2.3 Bacterial techniques

2.3.1 Preparation and transformation of competent cells

TSS transformation The method of Chung *et al.* (1989) was routinely used. A fresh overnight culture of the appropriate bacterial strain was diluted 1 in 100 into fresh L-broth and grown, with good aeration, to an OD_{600} of between 0.3 and 0.4. The culture was chilled on ice, transferred to a universal bottle and centrifuged at 4000 r.p.m. for 10–15 min in a bench centrifuge. The supernatant was removed and the bacterial pellet resuspended in 0.1x the original volume of ice-cold TSS buffer. At this point the cells could be frozen at -70°C, or could be used immediately for transformation. The plasmid DNA (typically 1–100 ng in <10 µl) was added to 0.1 ml of competent cells, mixed gently and stored on ice for 15–30 min. After this time, 0.9 ml of LBG (L-broth containing 20 mM glucose) was added and the cells incubated at an appropriate temperature for 1 h to allow expression of plasmid borne antibiotic-resistance genes (expression time was not necessary for tetracycline resistance). Two-hundred microlitres of this mixture was then spread onto antibiotic-containing plates and incubated until colonies appeared. An aliquot of competent cells lacking plasmid DNA was plated with the same selection as a control.

Cells were also transformed with replicative form M13 DNA using this method. In this case the LBG step was omitted and 0.25 ml of M13-sensitive plating cells added to the transformed cells. The mixture was added to 3 ml of molten L-top agar, mixed gently and poured onto a L-agar plate which, once set, was incubated at 37°C overnight to give single M13 plaques.

TSS buffer:	Difco Bacto Tryptone	10 g
	Difco Yeast Extract	5 g
	NaCl	10 g
	PEG 3350	100 g
	$MgSO_4.7H_2O$	20 mM
	DMSO	50 ml
	PIPES Buffer pH 6.5	10 mM
	distilled water to 1 litre	

Transformation by electroporation For plasmids that were transformed poorly by the TSS method and for linear transformation of *recD* strains, electroporation was the preferred method. Five millilitres of a fresh overnight culture of the strain to be transformed was inoculated into 1 l of L-broth. This was grown at the appropriate temperature with vigorous shaking to an OD_{600} of 0.5–1. The cells were chilled on ice and then harvested by centrifugation at 5000 r.p.m. in a Sorvall Superspeed centrifuge, using a GSA rotor for 15 min at 4°C. The pelleted cells were resuspended in a total of 1 l distilled water; this was repeated using volumes of 500 and 100 ml distilled water. The cells were then resuspended in 20 ml of 10% (w/v) glycerol in a 30 ml glass Corex tube, centrifuged as above using a SS34 rotor and resuspended in a final volume of 2 ml of 10% (w/v) glycerol. Cells were then either aliquoted and stored at -70°C, or used directly for electroporation.

For electroporation, 80 µl aliquots of cells were chilled (or thawed) on ice. DNA was then added (1-10 µg in approximately 5 µl of TE) and the cells incubated on ice for a further minute. The cells and DNA were then transferred to a pre-chilled electroporation cuvette. Electroporation was performed using a Gene-Pulser™ fitted with a Pulse Controller (Bio Rad Laboratories ltd.). The 25 µF capacitor was charged to a potential of 2.5 kV and the Pulse Controller set at 200 Ω. The cuvette was pulsed once for a time constant of 4.5–5 ms (field strength 12.5 kV cm^{-1}). The cuvette was then removed from the chamber and the cells immediately resuspended in 1 ml of SOC. This cell suspension was incubated at an appropriate temperature for 1 h to allow expression of incoming antibiotic resistance and then dilutions were plated onto the appropriate media (expression time was not necessary for tetracycline resistance).

SOC:
- 2% Bactotryptone
- 0.5% Bacto yeast extract
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM $MgCl_2$
- 10 mM $MgSO_4.7H_2O$
- 20 mM glucose

2.3.2 Frozen storage of bacterial strains

It was found that strains of *E. coli* could be conveniently stored at -70°C without suffering a dramatic loss of viability, including strains harbouring plasmids that might otherwise be lost. A fresh 5 ml overnight culture was prepared with antibiotic selection if required. This was centrifuged at 4000 r.p.m. for 10–15 min in a bench centrifuge, the supernatant discarded and the cells resuspended in 0.1x the original volume of Frozen Storage Buffer. The cells were then left on ice for a couple of hours before storing at –70°C.

Frozen Storage Buffer: 50% Bacterial buffer
50% glycerol (v/v)

2.3.3 Testing UV sensitivity of recA strains

A single colony of the strain to be tested was streaked across an L-agar plate using a sterile toothpick. In addition, single colonies of a known *recA* strain and a wild-type control were streaked across the plate to act as controls. The plate was covered with a piece of card and exposed to successively longer periods of UV irradiation along the streaks; commonly 0, 10, 20, and 40 sec under an UV lamp calibrated at 600 ergs mm^2 sec^{-1}. The plate was then incubated overnight. Growth of streaks was then compared for the different time intervals, and an estimate of UV sensitivity made. *RecA* strains tend to be sensitive to a 10 sec dose of UV. When checking the UV sensitivity of *recA*^{ts} strains it was necessary to carry out the above protocol on plates preheated to the non-permissive temperature and essential to ensure that their temperature did not drop significantly during the procedure.

2.3.4 Sizing and Counting of Bacterial Cells

A Coulter counter 2B (Coulter electronics) and Coulter channelyser model C-1000 were used for determining the size and number of bacterial cells. Cultures were grown in L-broth or Nutrient broth and 100 µl samples were added to an equal volume of filtered Fixing Solution. The sample could then be stored indefinitely in a sealed tube but cell-size and number determination were routinely carried out within 3–5 days of sample preparation. For measurements, a

noted volume of the sample was diluted with 6 ml of filtered Counting Buffer and mixed in a glass vial. The electrode was lowered into the vial and the sample read. The counter counts the number of cells in a fixed volume and the channelizer enables the cells to be placed into separate channels according to their size. From this distribution of different sizes, the mode and median cell size of a culture could be determined.

Fixing Solution	80% Bacterial buffer 20% Formaldehyde (v/v)
Counting Buffer	0.85% NaCl (w/v) 0.08% Sodium Azide (w/v)

2.3.5 Photography of bacterial cells

Cells were routinely photographed using a Zeiss photo-camera. About 1 ml of newly molten L-agar was pipetted on to a glass slide (washed in ethanol and dried) and any excess was quickly removed with the pipette. This was allowed to set on a level surface and then 10 µl of cell culture was spotted on to the agar. A coverslip (washed in ethanol and dried) was laid on top of this and the cells were photographed through a 100x, phase contrast, oil-immersion lens.

Visualisation of nucleoids. Nucleoids were visualised by the 'Fluo-Phase' method of Hiraga *et al.* (1989). Nucleoids were condensed by adding chloramphenicol (final concentration 200 µg ml^{-1}) to an aliquot of culture and then this was incubated at 42°C for 10 min, giving an immediate block to cell division. The cells were then centrifuged in a microfuge for 1 min and the supernatant removed. The pellet was washed once, by resuspension and centrifugation in bacterial buffer and then resuspended in an appropriate volume of bacterial buffer (depending on the density of the original culture). Ten microlitres of this suspension was immediately spotted onto a glass slide (previously washed in ethanol and dried) and allowed to air-dry. When dry, the spot was fixed by placing the slide in methanol for 5 min and allowed to air-dry. The slide was then ready for use or storage. Ten microlitres of a 1.5 µg ml^{-1} solution of 4,6-diamidino-2-

phenylindole (DAPI), was laid onto the spot of cells, a coverslip (previously washed in ethanol and dried) was applied and the nucleoids could be viewed and photographed under mixed phase and fluorescence illumination with a Leitz Metallux II microscope.

2.4 Phage techniques

2.4.1 Preparation of λ plate lysates

Cells were grown in L-broth containing 20 mM $MgSO_4.7H_2O$ and 0.2% maltose (v/v) at an appropriate temperature until they reached mid-log phase. Two hundred microlitre aliquots were then mixed with 10^6 λ phage, incubated at 37°C for 5 min, and 3 ml of molten L-top agar containing 20 mM $MgSO_4.7H_2O$ and 0.2% maltose (v/v) was added. This was poured onto a L-agar plate, left to set and incubated at 37°C overnight or until visible lysis occurred. Five millilitres of phage buffer was then added to the plate and the layer of top agar scraped off into a sterile 250 ml beaker. A few drops of chloroform were added and the beaker was incubated at room temperature with gentle swirling for 20 min. The contents of the beaker were poured into a universal bottle and centrifuged at 4000 r.p.m. for 10 min in a bench centrifuge. The supernatant was transferred to a fresh half-ounce bottle and stored over a few drops of chloroform at 4°C.

2.4.2 Preparation and selection of λ lysogens

A lawn of the bacterial strain to be lysogenized was made by mixing 0.2 ml of a mid-log phase L-broth culture and 3 ml of molten L-top agar with 20 mM $MgSO_4.7H_2O$ and 0.2% maltose (v/v). To this, approximately 200 λ phage particles were added, and the mixture poured on a fresh L-agar plate. Once set, the plate was incubated overnight at 37°C. This should result in the appearance of isolated λ plaques. The centre of a plaque was then touched with a sterile toothpick and streaked out onto another plate, which again was incubated overnight. The resulting single colonies could be tested for the presence of the λ phage. A lysogenized bacterium would now be resistant to lysis by phages with the same immunity as the one used to lysogenize the strain, but would be sensitive to λ phages that are virulent, or carrying a different immunity. This was tested by cross streaking single colonies of possible lysogens with the original λ phage used for the lysogenisation and λ*vir* as a control.

2.4.3 Preparation of Kohara phage lysates and phage DNA

Kohara phage are *red*$^-$, *gam*$^-$. Therefore these phages were amplified using strain DL307 (*recD*) as a host. A fresh overnight culture of DL307 was used to inoculate 200 ml of L-broth supplemented with 10 mM $MgSO_4.7H_2O$, and then incubated at 37°C with vigorous shaking. The OD_{600} of the culture was followed until it reached about 0.5, then the appropriate phage lysate was added to a multiplicity of infection (m.o.i.) of between 0.1 and 1. The OD was again followed until it began to drop, usually between 90 minutes and 4 hours after addition of the phage. At the culture's lowest OD, chloroform was added (approximately 0.5 ml for a 200 ml culture) and the flask left to shake for a further 10 min. Then 8 g of NaCl was added and left to dissolve. This was followed by adding DNAse and RNAse each to a final concentration of 1 ug ml^{-1} and the culture was left at room temperature for one hour. The solution was then transferred to a 250 ml centrifuge bottle and spun in a Sorvall Superspeed centrifuge using a GSA rotor at 10000 r.p.m. for 10 min. The supernatant was decanted into a 500 ml flask containing 20 g of PEG6000, and then left at 4°C overnight. The precipitate containing macromolecules and phage debris was precipitated by centrifugation as above at 10000 r.p.m. for 10 min. The supernatant was totally removed by first decanting and then using a pasteur pipette; this was discarded. Five millilitres of phage buffer was added and this was left to shake gently at 4°C until the pellet had totally disaggregated. To ensure complete resuspension the solution was taken up and expelled from a pasteur pipette several times. This was then centrifuged at 5000 r.p.m. for 10 minutes in a bench centrifuge. The supernatant was transferred to a sterile bottle and now this concentrated lysate could be purified by CsCl gradient centrifugation. Solutions of CsCl were prepared with densities of 1.3, 1.5 and 1.7 g ml^{-1}. A step-gradient was set up in a 14 ml polypropylene centrifuge tube, by layering 2 ml of first the 1.3 g ml^{-1} solution and then in turn the 1.5 and 1.7 g ml^{-1} solutions onto the bottom of the tube. The phage lysate was then added to the top of the gradient and centrifuged in a Sorvall OTD Ultracentrifuge using an MSE 6x14 swinging bucket rotor at 30000 r.p.m. for 35 min. The phage formed an opaque band between the 1.3 and 1.5 g ml^{-1} layers and was collected through the side of the tube using a syringe and

needle. The concentrated phage was dialysed against two changes of TE and DNA was extracted as described previously (*2.2.2*).

2.4.4 Preparation of phage P1 plate lysates

Preparation of phage P1 plate lysates was as for phage λ (*2.4.1*) except that 10^6 phage were added to 1 ml of late-log phase cells and this was incubated at 37°C for 30 min prior to addition to the top agar. The maltose was omitted, and the $MgSO_4.7H_2O$ was replaced with 2.5 mM $CaCl_2$. The phage buffer was also replaced with the same volume of L-broth containing 2.5 mM $CaCl_2$.

2.4.5 Mutagenesis of phage P1 lysates

In order to perform localised random mutagenesis of *E. coli* strains, a technique of phage P1 mutagenesis devised by Hong and Ames (1971) and adapted by Salmond *et al.* (1992) was used. A high-titre phage P1 lysate was made as above and the following mixture prepared in a 15 ml glass Corex tube:

P1 lysate	0.5 ml
phosphate-EDTA buffer (pH 6.0)	1 ml
distilled water	1.5 ml
hydroxylamine solution (freshly prepared)	2 ml

This was incubated at 37°C for 24 h and phage particles were then precipitated at 4°C in a Sorvall Superspeed centrifuge using a SS34 rotor at 17000 r.p.m for 2.5 h. The supernatant was removed and phage particles were recovered from the pellet by adding 0.5 ml phage buffer and allowing slow resuspension overnight.

phosphate-EDTA buffer (pH 6.3)	K_2HPO_4 (1 M)	13.2 ml
	KH_2PO_4 (1 M)	86.8 ml
	EDTA (10 mM)	100 ml
hydroxylamine solution	NH_2OH	0.35 g
	NaOH (4 M)	560 µl
	distilled water to 5 ml	

2.4.6 Phage P1-mediated transduction

A standing culture of the recipient strain of *E. coli* was grown up overnight in L-broth. The cells were harvested by centrifugation and the bacterial pellet resuspended in 0.1x the original volume of L-broth containing 2.5 mM $CaCl_2$. One hundred microlitre aliquots of this 10x culture were mixed with either 0.1 ml of phage P1 stock or 0.1 ml of a 10x dilution of the phage stock. These were incubated at 37°C for 15 min. If prototrophic transductants were to be selected, 0.4 ml of phage buffer was added and 0.2 ml aliquots of the cells plated on the appropriate minimal media agar plates. If the selection was for the acquisition of antibiotic resistance then 1 ml of LB containing 0.01 M sodium citrate was added. This was incubated at 37°C for 1 h to allow expression of the antibiotic resistance and then 0.2 ml aliquots were plated out on L-broth agar plates containing the appropriate antibiotic (expression time was not necessary for tetracycline resistance). Plates were incubated at a suitable temperature until colonies appeared.

2.5 Protein Techniques

2.5.1 Preparation of protein samples from cell cultures

Overproduction of proteins Overproduction of protein from a particular gene for visualisation by polyacrylamide gel electrophoresis was carried out by cloning the gene into pUC18 or pUC19 such that it would be expressed from the inducible *lac* promoter. A fresh overnight culture of cells containing the clone was diluted 1 in 100 into L-broth with the appropriate selection and grown at 37°C to an OD_{600} of about 0.5. Half a millilitre of this culture was transferred into an Eppendorf tube and isopropyl-β-D-thiogalactopyranoside (IPTG) added to a final concentration of 120 μg ml^{-1}. Incubation was continued at 37°C for 3 h. The induced cells were pelleted in a microfuge for 1 min and resuspended in 1x Sample Buffer (Laemmli, 1970). This solution was vortexed vigorously for 1 min and then incubated at 37°C for 1 h or at 100°C for 5 min (some proteins will not enter the gel matrix after treatment at 100°C). Denatured protein samples were then centrifuged for 5 min in a microfuge and 30 μl aliquots were loaded onto an acrylamide gel (*2.5.2*).

Specific radioactive labelling of proteins. When specific radioactive labelling of proteins was desired, the T7 system was used, (Studier and Moffatt, 1986, Tabor and Richardson, 1988). This system is extremely useful for the identification of proteins from genes which are difficult to express and labels proteins more specifically than methods using 'maxi-cells' or mini-cells. Genes to be overexpressed were cloned directly downstream of the ø10 promoter in one of the available vectors and transformed into strain BL21(DE3). A fresh overnight culture of a transformant was diluted 1 in 100 into minimal medium and grown at 37°C to an OD_{600} of about 0.8. Half a millilitre of this culture was transferred into an Eppendorf tube and expression of the T7 RNA polymerase was induced by the addition of IPTG to a final concentration of 120 μg ml^{-1} and incubation continued at 37°C for a further 30 min. Production of host proteins was inhibited by the addition of rifampicin to a final concentration of 200 μg ml^{-1} and incubation at 37°C for 45 min. Plasmid encoded proteins were then pulse–labelled by the addition of 5 μCi [^{35}S]-

methionine and incubation for 5 min. Cells were pelleted in a microfuge for 1 min and resuspended in 1x Sample Buffer. This solution was vortexed vigorously for 1 min and then incubated at 37°C for 1 h or at 100°C for 5 min. Denatured protein samples were then centrifuged for 5 min in a microfuge and 30 µl aliquots were loaded onto an acrylamide gel (*2.5.2*).

Molecular weight markers, obtained commercially (BDH Ltd., Poole, UK) were used in all gels. These consisted of Cytochrome C (12.3 kD), Myoglobin (17.2 kD), Carbonic Anhydrase (30 kD), Ovalbumin (42.7 kD), Albumin (66.25 kD) and Ovotransferrin (76–78 kD) and were denatured in Sample Buffer as for other samples.

2x Cracking Buffer	4x stacking gel buffer (*2.5.2*)	125 µl
	20% SDS	150 µl
	50% glycerol	200 µl
	β–mercaptoethanol	50 µl
	0.1% bromophenol blue	200 µl
	0.1% xylene cyanol	200 µl
	Distilled water	75 µl
	Total	1.0 ml

2.5.2 Polyacrylamide gel electrophoresis of proteins

Proteins were routinely separated using SDS–polyacrylamide gel electrophoresis with a discontinuous buffer system (Laemmli,1970). In virtually all cases a 10% resolving gel and 4% stacking gel were employed. An SE600 apparatus manufactured by Hoeffer Scientific Instruments was used, which gives gels measuring 11 x 14 cm. The following solutions were made up on ice immediately prior to use, with the ammonium persulphate solution and the TEMED being added last.

10% Resolving gel.

acrylamide stock solution (40%)		6.25 ml
4x resolving gel buffer		6.25 ml
10% SDS		0.25 ml
7.5% ammonium persulphate (w/v, freshly prepared)		0.25 ml
distilled water		12 ml
TEMED		15 µl
	Total	25 ml

4% stacking gel.

acrylamide stock solution (40%)		1.0 ml
4x stacking gel buffer		2.5 ml
10% SDS		0.1 ml
7.5% ammonium persulphate (w/v, freshly prepared)		0.1 ml
distilled water		6.3 ml
TEMED		10 µl
	Total	10 ml

The resolving gel solution was pipetted between the glass plates separated by 0.75 mm spacers; enough room was left for the stacking gel. The depth of the stacking gel between the bottom of the comb and the resolving gel was about 2.5 cm. Once the resolving gel had been poured, it was layered with *iso*-butanol saturated with 1x resolving-gel buffer and allowed to polymerise for 1 h. The isobutanol was then discarded and the top of the gel was washed several times with distilled water. The stacking gel solution was poured on top of the resolving gel, the comb inserted and polymerisation allowed to occur. The comb was then removed and the wells washed out with 1x reservoir buffer, which was also used to fill up the buffer chambers of the apparatus. The sample could be loaded onto the gel at this stage.

Protein samples were loaded using a Gilson P200 pipette. Gels were typically electrophoresed at a constant current of 40 mA until the bromophenol blue dye-front had reached the bottom of the gel. Once electrophoresis was complete the glass plates were removed

from the apparatus, separated carefully using a plastic wedge and the gel placed in staining solution for 45–60 min with constant gentle agitation. Gels were then transferred into destaining solution and left for 2h. For preservation, the stained/destained gel was soaked in destaining solution plus 5% glycerol for 30 min and dried down on blotting paper using a vacuum gel drier at 80°C for about 1 h.

2.5.3 Solutions used in SDS–PAGE

Stock acrylamide

30 g acrylamide, 0.8 g NN' methylene bis–acrylamide, made up to 100 ml with distilled water, filtered and stored at 4°C.

4x stacking-gel buffer (0.5 M Tris)

15.25 g of Tris base, dissolved in 200 ml distilled water, adjusted to pH 6.8 with concentrated HCl, made up to 250 ml, filtered and autoclaved.

4x resolving-gel buffer (1.5 M Tris)

45.5 g of Tris base, dissolved in 200 ml distilled water, adjusted to pH 8.8 with concentrated HCl, made up to 250 ml, filtered and autoclaved.

10x reservoir buffer

30.2 g of Tris base, 144 g of glycine dissolved in 600 ml distilled water, made up to a final volume of 1 litre and filtered. SDS was added to 0.1% in the final 1x buffer.

Staining solution

9% (v/v) acetic acid, 45% (v/v) methanol, and 0.1% (w/v) Coomassie brilliant blue.

Destaining solution

7% (v/v) acetic acid, and 5% (v/v) methanol.

CHAPTER 3

PARTITIONING IN SPHERICAL CELLS AND CHARACTERISATION OF A 'PARTIAL DIPLOID' STRAIN

3.1 Introduction

It has been established that in cultures of spherical *pbpA* or *rodA* mutants growing at the same rate as otherwise isogenic rod-shaped strains, the spherical cells have approximately four times the volume of the rod-shaped cells. They also have approximately four times as much DNA as the rod-shaped cells (Donachie and Begg, 1989). This infers that the spherical cells are polyploid. It appears therefore, that partitioning in spherical cells may be a more complex task than in rod-shaped cells. For example, in the spherical equivalent of a rod-shaped cell with two chromosomes at division, eight chromosomes must be partitioned. Another consequence of being spherical is that partitioning is not constrained spatially by the narrow lumen of rod-shaped cells. These properties of spherical cells allow questions about the nature of partitioning to be asked which may not arise, and are not experimentally testable in rod-shaped cells. It is reasonable to assume that a cell does not develop an entirely new system of partitioning reserved solely for when it is spherical. Therefore, it is proposed that experiments designed to study partitioning in spherical cells may shed more light on the nature of the process in wild-type, rod-shaped cells. In short, it may be helpful to determine exactly *what* happens to chromosomes during partitioning, before finding out exactly *how* it happens.

3.1.1 Possible modes of partitioning in spherical cells

Partitioning is random. It has been proposed that partitioning is directly associated with the cell-wall (or membrane) and that the lumen of the rod-shaped cell automatically determines an axis along which chromosome separation occurs (*e.g.* Jacob, Brenner and Cuzin, 1963, Cook *et al.*, 1987). If this is the case, then it is possible that the dramatic morphological change of a cell from a rod to a sphere would cause that axis to disappear rendering the partitioning apparatus useless. In such a cell the partitioning of chromosomes would be, at best, random. Spherical mutants *were* detected in assays for partitioning defects carried out by Hiraga *et al.* (1989), see Chapter 1, however the appearance of DNA-less cells was due to chromosome degradation rather than partitioning defects *per se* (S

Hiraga *personal communication*). It appears therefore, that partitioning is not detectably worse in spherical cells than in rod-shaped cells.

It remains a formal possibility however, that the process itself is actually random, that is, there is no partitioning apparatus in *E. coli* (Figure 3.1.1). In rod-shaped cells, which normally contain two complete chromosomes at division, random partitioning might not manifest itself experimentally, due solely to the relative dimensions of the cell and chromosomes ensuring that there is always one chromosome either side of the septum at division. The isolation of apparent partitioning mutants isolated by Hiraga *et al.* (1989) and the well-documented regular distribution of condensed nucleoids in filamenting cell-division mutants (for example see Figure 4 in Begg and Donachie, 1991 and Figure 5.3.5 in this thesis) argues against this suggestion, however neither totally rules out random partitioning.

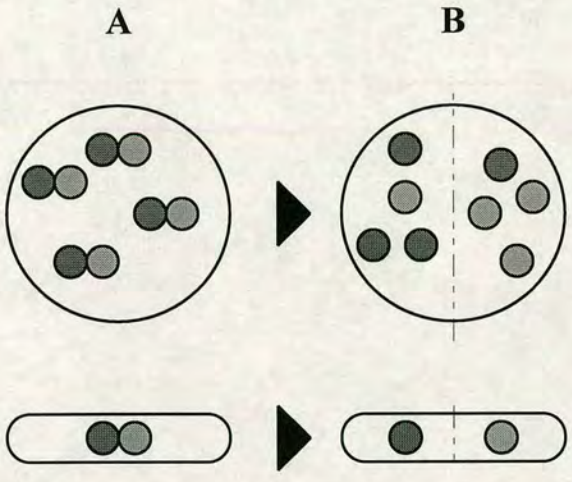


Figure 3.1.1 *Random partitioning of chromosomes in spherical and rod-shaped cells.* Shaded circles indicate single chromosomes. Dotted lines indicate the plane of the next division. **A:** shows the cells immediately prior to decatenation of sister chromosomes. **B:** shows the cells immediately before division. [If partitioning is random there will not necessarily always be equal numbers of chromosomes either side of the septum.]

The partitioning apparatus splits the total DNA in half. It is possible that an active partitioning process could have a random element to it. In studies comparing the growth of spherical and rod-shaped strains, Donachie and Begg (1989) found that the average number of condensed nucleoids per cell was the same for both. Hence a sphere containing eight chromosomes immediately before division would have two nucleoids, each consisting of four complete chromosomes. The situation illustrated in Figure 3.1.2 could therefore be envisaged; here the partitioning apparatus acts upon two nucleoids, thus crudely separating half of the total DNA into each daughter cell. This again could be reconciled with the regular nucleoid distribution observed in filaments, as follows; if partitioning is a linear event occurring over a fixed distance, as is often proposed (Donachie and Begg, 1989, Begg and Donachie, 1991, Hiraga *et al.*, 1990) a spherical cell with eight chromosomes (which is only as wide as the length of a rod with two chromosomes) may have room for only one partitioning event. In an equivalent filamentous cell with eight chromosomes there is room for four such events.

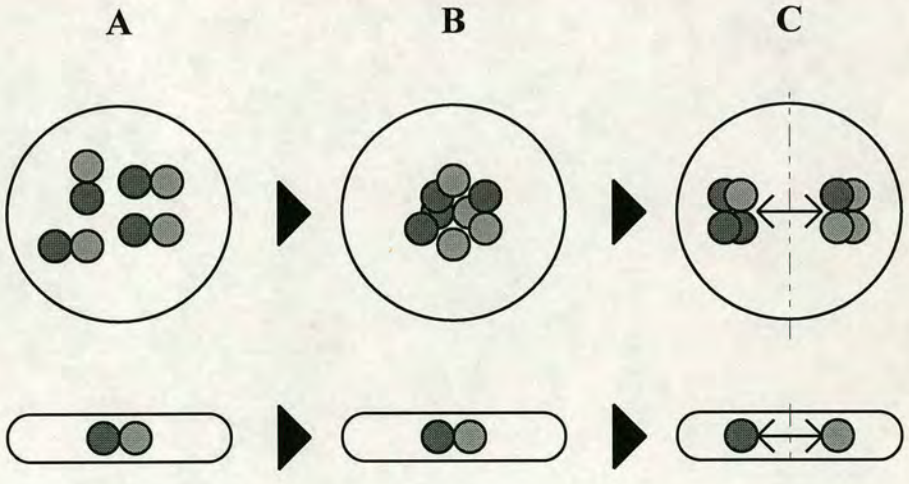


Figure 3.1.2 *Equipartition of two halves of the total DNA as nucleoids, in spherical and rod-shaped cells.* Shaded circles indicate single chromosomes. Dotted lines indicate the plane of the next division. Arrowed lines indicate the result of the action of the partitioning apparatus. **A:** shows the cells immediately prior to decatenation of chromosomes. **B:** shows the cells immediately prior to partitioning. **C:** shows the cell immediately after partitioning.

Partitioning is like mitosis. It is possible that bacterial chromosomes are partitioned by a 'mitotic' apparatus which ensures that each daughter chromosome is regimentally separated from its sister to opposite sides of the imminent septum. This implies the existence of a very specific cellular axis, perpendicular to the plane of division, along which all partitioning events would take place (perhaps the cell-spanning filaments visualised by over-expression of CafA, (Okada *et al.*, 1994); see Chapter 1). Again, this would be difficult to assess when considering a rod (which has an obligate axis determined by its shape) but the existence of such a system in spherical cells has more profound implications; see section 3.1.2. Mitotic partitioning is illustrated in Figure 3.1.3. The equivalent of a mitotic apparatus in *E. coli* has often been looked for. Indeed, some interesting *E. coli* proteins have been found with similarities to important eukaryotic structural or force-generating proteins, for example RNaseE (Casaregola *et al.*, 1990), FtsZ (RayChaudhuri and Park, 1992, deBoer *et al.*, 1992, Mukherjee *et al.*, 1993), MukB (Niki *et al.*, 1992) and CafA (Okada *et al.*, 1994); see Chapter 1.

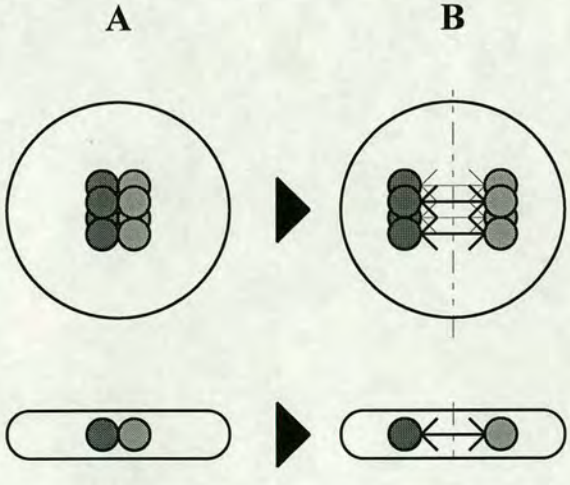


Figure 3.1.3 *'Mitotic' partitioning of chromosomes in spherical and rod-shaped cells.* Shaded circles indicate single chromosomes. Broken lines indicate the plane of the next division. Arrowed lines indicate the result of the action of the partitioning apparatus. **A:** shows the cells immediately prior to partitioning. **B:** shows the cells immediately after partitioning. Four partitioning events are shown in the sphere however perhaps one event could have the same specific effect.

Partitioning is vectorial along the cell perimeter. The apparatus for partitioning could operate by moving chromosomes apart by a fixed distance, with the cell-wall or membrane acting to define the movement. Again, in a normal rod this would manifest itself in the same way as any of the previous suggestions. The regular nucleoid distribution observed in filaments supports the idea that this *does* happen (at multiple sites) but the direction of movement would still be restricted by the narrow lumen of the cell. In a spherical cell this is not the case, so one could imagine at least two possible situations: [i] the direction of movement is defined by a more subtle property of the cell-wall or membrane than its shape (for example, the orientation of peptidoglycan strands), leading to ordered partitioning and positioning of chromosomes comparable to that observed in filaments; [ii] the partitioning apparatus needs to be anchored at the cell perimeter but its direction is normally determined by nothing more specific than the narrow lumen of rod-shaped cells. In a spherical cell the direction of partitioning would therefore be unconstrained except by the presence of adjacent chromosomes. These ideas are illustrated in Figure 3.1.4.

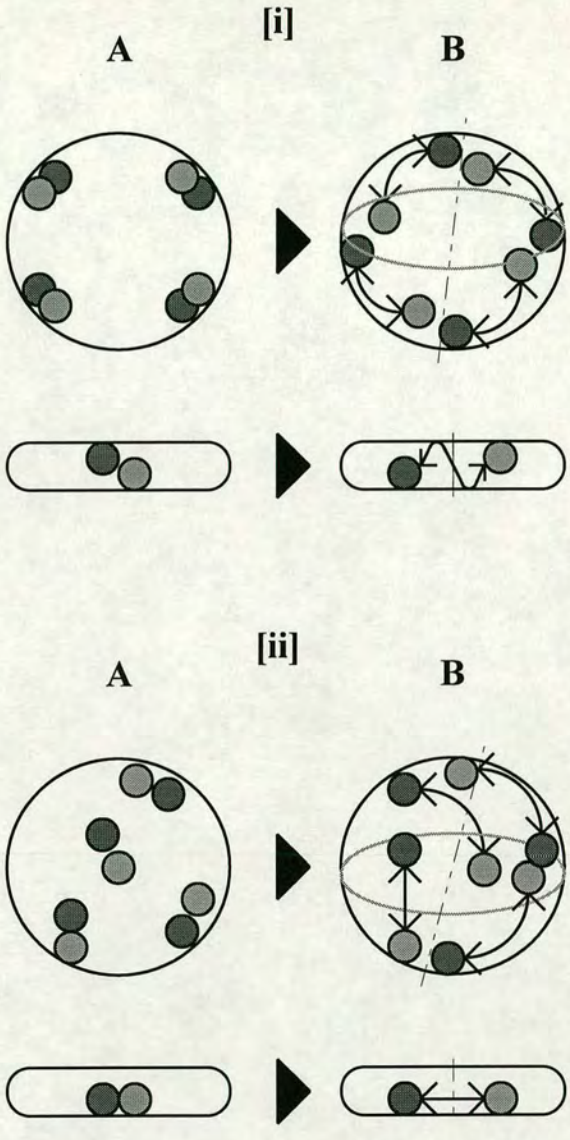


Figure 3.1.4 *Perimeter-mediated partitioning in spherical and rod-shaped cells.* Broken lines indicate the plane of the next division. Arrowed lines indicate the partitioning apparatus driving the chromosomes along the wall or membrane. [i] The direction and orientation of partitioning is identical in all pairs of chromosomes due to an integral property of the wall or membrane. [ii] The direction and orientation of partitioning is random but still wall or membrane-mediated. **A:** shows the cells immediately prior to partitioning. **B:** shows the cells immediately after partitioning. [The random aspect to [ii] means that there will not necessarily be equal numbers of chromosomes either side of the septum at each division.]

3.1.2 Experimental rationale

Having postulated that there are a number of possible ways in which the cell could partition its chromosomes the following approach was taken to try to distinguish between them. It was proposed that the polyploidy of spherical cells could be exploited by making one of the chromosomes genetically distinct from the others, that is, making a chromosomal heterozygote. The segregation behaviour of the two different types of chromosomes would allow conclusions to be drawn about the nature of the partitioning process. The validity of this approach is supported by experiments in *Deinococcus* (formerly *Micrococcus*) *radiodurans* which contains between four and ten separate but identical chromosomes. It has been shown to be transformable but only after a period of segregation (Hansen, 1978, Tigari and Moseley, 1980, Masters *et al.*, 1991). Another bacterial species, *Azotobacter vinelandii* was previously thought to have up to 80 independently segregating chromosomes (Punta *et al.*, 1989). A recent paper suggested that this may not be true due to the appearance of incoming recessive markers after only seven generations (Moldanao *et al.*, 1992). It was concluded that these bacteria were mildly polyploid (Moldanao *et al.*, 1992); see Discussion (*3.3.2*).

The possible ways in which partitioning in spherical cells could occur give rise to different predictions about the results of such experiments. If partitioning was mitotic in nature (Figure 3.1.3), then genetic alteration of one of the chromosomes would result in a permanently heterozygous cell, due to the absolute separation of each chromosome from its sister at every division, leaving all progeny with an essentially identical complement of chromosomes. In a cell where partitioning is random (Figure 3.1.1) or has any random element to it (Figures 3.1.2 and 3.1.4[ii]) then genetically different chromosomes would segregate from each other at a characteristic frequency but it would be possible to maintain a heterozygote by selection. In Figure 3.1.4[i] the predicted segregation characteristics would depend on the relationship between chromosome partitioning and the placement of the septum; see Discussion and Figure 3.3.3.

In the case of a rod-shaped cell this experiment would show immediate segregation of the two different chromosomes at the first division, regardless of the nature of the partitioning process.

3.2 Results

3.2.1 Initial experiments on partitioning in spherical cells

The chosen method of genetic transfer was phage P1-mediated transduction (*2.4.6*).

Transduction with single selection. Initially, strain KJB24 (a spherical version of strain W3110, due to a *rodA* amber mutation in a sup^0 background) was transduced with a P1 lysate of strain SHA2 (*leu*::Tn*10* at 2 minutes on the *E. coli* chromosomal map) and plated with selection for tetracycline resistance (Tet^R). A normal, haploid Tet^R transductant would become auxotrophic for leucine (leu^-). A cell able to remain heterozygous after transduction into one chromosome would become Tet^R but remain prototrophic due to uninterrupted copies of the leucine gene on other chromosomes. Transductant colonies were screened for these characteristics by patching onto M9 minimal plates as follows; M9 + leucine (M9L), M9 only (M9), M9 + Tet + leucine (M9TL) and M9 + Tet (M9T). All transductants from this kind of experiment were found to be Tet^R, leu^-. The reciprocal experiment was carried out; a spherical Tet^R leu^- strain from the first transduction (SHA4) was transduced with a P1 lysate made on a leu^+ strain (W3110) and plated with selection for leu^+. All transductants from this kind of experiment were found to be Tet^S, leu^+. The two experiments were carried out on equivalent rod-shaped strains (W3110 and SHA2 respectively) with identical results. These results appeared to immediately rule out the possibility of mitotic partitioning.

[In a repetition of the transduction of KJB24 to Tet^R, KJ Begg isolated a colony which appeared to have both Tet^R and leu^+ markers. The characterisation of this (exception number 1, strain SHA5) is described in section 3.2.2.]

Transduction with double selection. The following experiments were designed to look for the segregating heterozygotes predicted for random or semi-random partitioning. Identical transductions to those described above (**A:** KJB24 x P1[SHA2], **B:** W3110 x P1[SHA2], **C:** SHA4 x P1[W3110] and **D:** SHA2 x P1[W3110]) were repeated, except

selection was for heterozygous (Tet^R, *leu*$^+$) transductants. That is, all transductions were plated on M9T. Aliquots were plated onto single selective plates (LB + tetracycline (LBT) for A and B, M9 for C and D) to check the transduction frequency.

Three spurious colony-types resulted from these transductions. Occasionally large, mucoid colonies appeared; they seemed to be able to survive the tetracycline, protected perhaps by excessive surface secretions but this was apparently not a genetic trait, as they were Tet^S when subsequently streaked on LBT plates. This type of colony was rare and did not appear in every transduction. They were no more frequent in spherical strains than rod-shaped strains and were consequently discounted from further analysis. Colonies often appeared in transductions A and B which, although they appeared on a M9T plate, were found to be Tet^R, *leu*$^-$. These were apparently 'normal' transductants which were able to form colonies on a M9T plate without having the leucine gene. Either these transductants had sufficient leucine biosynthetic enzymes remaining from the *leu* gene (removed by transduction) to allow them to produce a colony, or they originally contained a large pool of leucine. This type of colony did not appear in transductions C and D (where the *leu*$^+$ gene was replacing the *leu*::Tn*10*) leading to the following conclusions. [i] The presence of a large pool of leucine should not differ between KJB24 and SHA2 grown in LB, therefore the former explanation for the production of these colonies was more likely. [ii] When the Tet^R gene was removed by transduction the enzyme did not appear to linger in the same way as above because no spurious colonies of this type appeared. This ultimately meant that selection against Tet^S colonies was stronger than selection against *leu*$^-$ colonies. Very occasionally Tet^R *leu*$^+$ colonies were isolated. In all but one of these the Tet^R transposon was no longer associated with the leucine auxotrophy; it had moved elsewhere. The one exception (exception 2) is described in the next section. The important point from these experiments is that it was very difficult to isolate a heterozygous clone, suggesting that random partitioning in spherical cells was also improbable.

Strain LM1 - exception number two. A colony (strain LM1) isolated from a repetition of transduction C belonged to none of the three

spurious colony-types mentioned above. LM1 was isolated on a M9T plate and so appeared to be Tet^R, *leu*$^+$. It was immediately streaked to single colonies on three selective plates (M9, M9TL, M9T) and incubated overnight. Growth on the M9T plate confirmed that it was Tet^R, *leu*$^+$. Ten colonies from each plate were patched out onto a similar set of plates and incubated overnight; the results are shown in table 3.2.1. The experiment tested the idea that a randomly segregating heterozygote would be selectively maintainable on M9T plates, but selection for one marker by itself might allow the other to be lost by random segregation.

Table 3.2.1 Initial segregation characteristics of strain LM1

		Colonies patched onto:		
		M9T	M9TL	M9
Colonies taken from:	M9T	+	+	+
	M9TL	-	+	-
	M9	-	-	+

\+ 10/10 patches grew
\- 0/10 patches grew

It appeared that LM1 remained Tet^R, *leu*$^+$ if plated on M9T but the markers segregated very quickly if one selection was relaxed; it became *leu*$^+$ if streaked on M9TL and became Tet^S if streaked on M9. The streaking and patching was then repeated four times, each time with at least three Tet^R, *leu*$^+$ colonies of LM1 (it was found that only some of these colonies still segregated; stable colonies were ignored in the table below, but see later). A fourth, non-selective plate (M9L or LB) was also used in two of these experiments; a collation of the results from the four experiments are shown in table 3.2.2.

Table 3.2.2 Segregation characteristics of strain LM1

		Colonies patched onto:			
		M9T	M9TL	M9	M9L
Colonies taken from:	M9T	93	90	99	96
	M9TL	8	100	8	99
	M9	6	5	100	100
	M9L	6	71	77	100
		% positive patches			

The segregation pattern was essentially the same as shown in table 3.2.1, when LM1 was first isolated but, by patching more colonies, it was shown that markers did not segregate completely in every clone. When there was no selection at all, some Tet^R, *leu*$^+$ clones persisted but segregation was frequent and happened approximately equally in either direction. The strain LM1 therefore appeared to have the predicted properties of a segregating chromosomal heterozygote and supported the idea that partitioning was random. However there were some worrying features that placed doubts on this conclusion.

[i] LM1 was an exception to the rule. If partitioning was always random then many (if not all) transductants should have similar properties. Other strains like LM1 have not been isolated during many repetitions of the same experiment. The equivalent experiment has also been carried out with *thr*::Tn*10* with equal lack of success.

[ii] Segregation was happening very quickly such that more than 90% of all colonies had totally lost one of the markers between streaking and patching when one selection was omitted (table 3.2.2).

[iii] LM1 had a tendency to stabilise, resulting in colonies from which no segregants were produced at all, even when it was stored at -70°C in frozen storage buffer.

Characteristics [i] and [ii] of LM1 were similar to strain SHA5 (exception number 1). Because of these similarities, the next section (*3.2.2*) describes the characterisation of SHA5. Also, the rationale for further transduction experiments, based on the ones discussed above, came directly from the characterisation of SHA5. These experiments will be discussed in section 3.2.3.

3.2.2 Characterisation of SHA5, a 'partial-diploid' strain

A Tet^R, *leu*$^+$ strain was isolated by KJ Begg from a transduction of KJB24 (on to LBT), identical to transduction A described in section

3.2.1. The strain appeared to be a possible chromosomal heterozygote and so was investigated.

Detection of segregation. When SHA5 was streaked out onto non-selective media, the Tet^R and leu^+ alleles were found to segregate from one another at a low frequency. Segregation was detected by patching single colonies onto M9L, M9, M9TL, M9T plates, as before and the results were documented by classifying each colony according to its phenotype. The classification of phenotypes is defined in table 3.2.3. [Because of its unstable nature, SHA5 was routinely maintained on M9T plates; frozen storage buffer was used for long term storage.]

Table 3.2.3 Classification of colony phenotypes, based on patching results

		Colonies patched onto:			
		M9T	M9TL	M9	M9L
Phenotype:	Tet^R leu^+	+	+	+	+
	Tet^R leu^-	-	+	-	+
	Tet^S leu^+	-	-	+	+

\+ growth
\- no growth

The segregation characteristics of SHA5 are shown in table 3.2.4. The values represent the percentage of colonies which are of each phenotypic class after overnight streaking on LB from a single colony on M9T. This data was collated from seven separate streakings involving 470 single colonies.

Table 3.2.4 Segregation characteristics of SHA5

	Phenotype:		
	Tet^R leu^+	Tet^R leu^-	Tet^S leu^+
% of colonies (n=470):	84	8	7

The segregation was approximately equal in either direction, however it was impossible to distinguish between whether the segregation occurred in the colony on the M9T plate or only happened when the selection was relaxed. To test whether segregation could be directed

by a particular selection, SHA5 was streaked to single colonies on all four types of plate (M9L, M9, M9TL, M9T) and then patched as above. The data (including that from table 3.2.4) is displayed in table 3.2.5.

Table 3.2.5 Segregation characteristics of SHA5 grown on different media

		Phenotype:			[Number of
		Tet^R *leu*$^+$	Tet^R *leu*$^-$	Tet^S *leu*$^+$	colonies (n)]
Streaked onto:	M9T	96	1	3	246
	M9TL	78	20	2	50
	M9	84	6	10	50
	M9L	84	8	7	470
		% of colonies			

The most likely explanation for these results is that segregation of the two detectable markers was happening all the time, even in the colonies on M9T plates (as shown by the low but detectable frequency of segregants on the totally selective media). When there was no selective pressure on the overnight streaks, both kinds of segregant grew happily. When streaked on M9TL then the Tet^R *leu*$^-$ segregants were more likely to survive. When streaked on M9 then the Tet^S *leu*$^+$ segregants were more likely to survive. The minor presence of the 'wrong' kind of segregants on the singly selective plates and *any* segregants on the totally selective plates, represented a similar situation to the appearance of spurious heterozygotes in the transduction experiments in section 3.2.1. The data in table 3.2.5 also supported the idea (*3.2.1*) that selection against Tet^S colonies was a stronger selection than against *leu*$^-$ colonies, that is, there were proportionally more 'wrong' segregants on M9 than there were on M9TL.

SHA5 contains both leu::Tn10 and leu$^+$ *alleles.* In section 3.2.1 it was mentioned that very occasionally the *leu*::Tn*10* transposon was found to have moved from its original position. This was discounted in SHA5 by the following experiment. A P1 lysate was made on SHA5 and this was used to transduce W3110 to Tet^R, *leu*$^-$ and to transduce SHA2 to *leu*$^+$. For transduction W3110 x P1[SHA5], 20/20 Tet^R transductants were *leu*$^-$. For transduction SHA2 x P1[SHA5], 50/50

leu$^+$ transductants were Tet^S. This experiment showed that both *leu*::Tn*10* and *leu*$^+$ alleles were present in SHA5 and that they could be transduced out of that strain separately.

Analysis of segregants. SHA5 was an exception to the general rule derived from section 3.2.1, that heterozygotes do not result from transduction of spherical cells. It was therefore proposed that SHA5 may have been a mutant which was genetically predisposed to more stable maintenance of two different chromosomes than wild type spherical cells. In order to test this proposal the mutant genetic trait was looked for in the two types of segregants produced from SHA5.

First these strains were tested to see if the allele that they had 'lost' could be being masked somehow. The Tet^S, *leu*$^+$ segregant (SHA6) and the Tet^R, *leu*$^-$ segregant (SHA7) were streaked on LB overnight and then patched onto the four selective media to see if their phenotypes were stable. 50/50 of each kept the same phenotype. P1 lysates were made of both and were found to be able to transduce only the allele of the leucine locus which they phenotypically displayed (the SHA6 lysate could transduce *leu*$^+$ only and the SHA7 lysate could transduce *leu*::Tn*10* only). This determined that the Tet^R allele had been lost from SHA6 and the *leu*$^+$ allele had been lost from SHA7. Another remote possibility was that segregants had appeared because they had become rod-shaped (and therefore unable to maintain two different chromosomes) but this was discounted by microscopic analysis. To test for the presence of a genetic trait favouring the maintenance of heterozygosity, SHA6 was transduced to *leu*::Tn*10* on LBT and screened for the *leu*$^+$ allele and SHA7 was transduced to *leu*$^+$ on M9 and screened for Tet^R. The results are shown in table 3.2.6.

Table 3.2.6 Phenotypes of transductants of SHA6 and SHA7

	Phenotype:		
	Tet^R *leu*$^+$	Tet^R *leu*$^-$	Tet^S *leu*$^+$
Number of SHA6 transductants:	59	19	0
Number of SHA7 transductants:	27	0	23

The main conclusion from these results was that a much higher proportion of the transductants were heterozygous (76% for SHA6 and 54% for SHA7) than in the original experiments (<0.2%), hence both SHA6 and SHA7 have inherited a trait which allows them to maintain heterozygosity. One of the SHA6 transductants (SHA8) was streaked out on LB overnight and segregation of a similar nature to that in SHA5 was detected. The results are shown in table 3.2.7.

Table 3.2.7 Segregation characteristics of SHA8

	Phenotype:		
	Tet^R *leu*$^+$	Tet^R *leu*$^-$	Tet^S *leu*$^+$
% of colonies (n=50):	94	2	4

Heterozygosity is not shape-dependent. The original aim of the experiment in which SHA5 was identified was to detect chromosomal heterozygosity in otherwise wild type spherical cells. The prediction was that this could not be successful in rod-shaped cells regardless of the nature of the partitioning process. The fact that SHA5 appeared to have a genetic propensity to remain heterozygous rather than only being that way due to selection, together with its extreme rarity, suggested that this strain's properties were not a good indication of the nature of partitioning. It was confirmed that SHA5 did not fit into the experimental model as defined in section 3.1.2 by the following experiments.

SHA5 was transformed with plasmid pSU66, which contained the *rodA* gene. SHA5 pSU66 transformants were rod-shaped due to complementation of the *rodA* amber mutation. The transformants were screened for maintenance of the heterozygous phenotype of SHA5. The results are displayed in table 3.2.8.

Table 3.2.8 Phenotypes of SHA5 pSU66 transformants

	Phenotype:		
	Tet^R *leu*$^+$	Tet^R *leu*$^-$	Tet^S *leu*$^+$
% of colonies (n=138):	52	46	2

The significant result here is that a large proportion of the transformants are still heterozygous. This means that heterozygous rod-shaped cells are easily produced, entirely contrary to the hypothesis put forward in section 3.2.1. The result supports the inheritance of a predisposition to remain heterozygous. Two of the SHA5 pSU66 transformants which were still heterozygous were screened for segregation as described above. Table 3.2.9 displays a collation of these results which show that SHA5 pSU66 segregated like SHA5 but at a much higher frequency.

Table 3.2.9 Segregation characteristics of SHA5 pSU66 heterozygotes

	Phenotype:		
	Tet^R *leu*$^+$	Tet^R *leu*$^-$	Tet^S *leu*$^+$
% of colonies (n=86):	49	20	31

It was possible to transduce both alleles separately from SHA5 pSU66 into recipient strains in exactly the same way as for SHA5. To complement the above experiments, SHA6 was also transformed with pSU66. Ten of these transformants were then transduced to Tet^R with a lysate containing *leu*::Tn*10* and then 25 colonies from each transduction were screened for heterozygosity. The number of heterozygous transductants detected for the ten different transformants was as follows: 0, 3, 0, 0, 0, 0, 0, 1, 0, 1 each out of 25. Comparing these with the proportion of SHA6 transductants which are heterozygous (76%, table 3.2.6) indicated that transformation of SHA6 with pSU66 destabilised the property which had been inherited from SHA5. This also helped to explain why SHA5 pSU66 heterozygotes segregated at a much higher frequency than SHA5 (see table 3.2.9).

SHA5 is a 'partial-diploid' strain. The results above, showing that SHA5 and its segregants had a genetic trait which allowed them to maintain heterozygosity, prompted the notion that there may be some property of the leucine locus in these strains contributing to their unexpected behaviour. Experiments were planned to see if other loci around the chromosome behaved in the same way as the leucine

locus. The segregation characteristics of similar pairs of alleles at other positions on the chromosome were to be investigated. This data was not obtained however, because it was found that these strains could not be made heterozygous for certain genetic loci, that is, some genes remained haploid. For example, SHA5 was transduced to chloramphenicol resistance (Cm^R) with a lysate of strain DL654 which is *recA*::Cm^R. 10/10 transductants were found to be *recA*$^-$, showing that the *recA* gene is haploid in SHA5. 5/10 of these transductants were Tet^R, *leu*$^+$ and therefore still diploid for the leucine locus. SHA6 was used for further characterisation of the diploid/haploid nature of these strains. Table 3.2.10 shows the combination of data from transductions carried out by KJ Begg and myself to determine the ploidy of a number of genes around the chromosome of SHA6.

Table 3.2.10 Ploidy of genes around the chromosome of SHA6

Gene	*thr*	*leu*	*mur F*	*pur E*	*nad A*	*rec A*	*arg A*	*arg G*	*ilv*	*arg E*	*rpo B*
Haploid (H) or Diploid (D)	D	D	D	D	H	H	H	H	H	H	H/D
Position (min)	0	2	2	12	14	58	60.5	69	85	89.5	90

These results show that SHA5 and SHA6 contain a large duplication of a region from at least 0' to 12' on the chromosomal map. Other genes around the 2' region of the chromosome were investigated in this way and *ftsQ*, *ftsA*, *ftsW* and *ftsZ* were all found to be diploid in SHA6. The *ftsI* gene was found to be haploid in SHA6, by this test, indicating that the region between 0 and 12 minutes on the chromosome might not be completely diploid in these strains. Subsequently SHA5 and derivatives were described as 'partial-diploid' strains.

The discovery that SHA5 had two copies of a large region of the chromosome containing the leucine locus helped to explain some of the phenomena encountered previously:

[i] Reciprocal segregation observed on LB plates, e.g. table 3.2.4, could be explained by reciprocal gene-conversion of the locus on one copy to the same as the locus on the other copy. That is, a chromosome containing *leu*::Tn*10* on one version of the 2' region and *leu*$^+$ on the other (SHA5) becomes a strain with *leu*$^+$ on both (SHA6) or *leu*::Tn*10* on both (SHA7). [The word reciprocal is used here to mean that this gene-conversion is equally likely to happen in either direction, resulting in equal numbers of each type of segregant.]

[ii] Retransduction of SHA6 with *leu*::Tn*10* (and SHA7 with *leu*$^+$) results in Tet^R, *leu*$^+$ cells because the transductions replace only one of the resident leucine alleles.

[iii] Rod-shaped cells can be diploid by inheriting the 'partial-diploid' chromosome for long enough for it to be detected. The haploid nature of most pSU66 (rod-shaped) transformants of SHA6 and the high segregation rate of transformants of SHA5 is due to the duplicated region of the chromosome being unstable in rod-shaped cells.

[iv] LM1 (*3.2.1*) was probably also a 'partial-diploid' strain except that its duplication was much less stable and was lost at a high frequency. It was mentioned in section 3.2.1 that LM1 produced frequent non-segregating diploids. [LM1 was so unstable that even after storage in Frozen Storage Buffer at -70°C no colonies with the same properties could be recovered.] This also occurred with SHA5 but it was much rarer. The nature of the stabilisation is unknown, however it could be an indication that the two strains are similar in nature except that the duplication in SHA5 is better tolerated than that in LM1.

Segregation of alleles in SHA5 and derivatives is recA-dependent. The characterisation of SHA5 and its derivatives led to the surprising result that *recA* (as well as other genes) was haploid in these strains. The segregation characteristics of a *recA* version of SHA5 (SHA36) were subsequently investigated. SHA36 was found not to produce segregants at all; 50/50 patches on each of the four types of media were Tet^R, leu^+. A heterozygous SHA5 pSU66 was also made *recA* giving the same result when screened for segregants. That is, *recA* versions of these strains were stable. This result suggested that both the process of segregation and the process of losing the chromosomal duplication in pSU66 transformants, were *recA*-dependent.

In order to further test this conclusion, an attempt was made to stimulate the production of segregants from a heterozygote by UV irradiation. The idea was that stimulation of *recA*-dependent DNA repair may stimulate the (apparently *recA*-dependent) segregation. A single colony of a Tet^R, leu^+ transductant of SHA6 (same as SHA8 - SHA8') from a M9T plate was streaked out onto two NB plates. One of these plates was irradiated for 15 s under an UV lamp calibrated at 600 ergs mm^2 sec^{-1}. The two plates were incubated at 37°C overnight and then 50 single colonies were screened for their phenotypes by patching onto M9L, M9, M9TL and M9T plates as before. At the same time, a toothpick was used to take cells from the confluent growth on the non-irradiated and irradiated plates. Each was streaked onto a single NB plate and the one from the irradiated plate was further irradiated for 15 s. The plates were incubated and single colonies screened as above. This was repeated twice more. Table 3.2.11 displays the results from this experiment. An extra column indicates the presence of mixed colonies; these give positive patches on both M9 and M9TL plates but not on M9T.

Table 3.2.11 Effect of UV irradiation on segregation of SHA8'

		Phenotype:							
		Tet^R *leu*$^+$		Tet^R *leu*$^-$		Tet^S *leu*$^+$		Tet^r *leu*$^-$ and Tet^S *leu*$^+$	
		-UV	+UV	-UV	+UV	-UV	+UV	-UV	+UV
Number of streak:	1	100	92	0	2	0	6	0	0
	2	100	84	0	8	0	8	0	0
	3	96	69	0	16	4	10	0	4
	4	98	58	2	14	0	28	0	0
		% of colonies							

The amount of segregation when the cells were UV irradiated was always significantly higher than when they were not. This supported the assertion that segregation was due to a *recA*-dependent recombination activity similar to those involved in *recA*-dependent DNA repair.

Implications of SHA5 characterisation on partitioning experiments. The characterisation of SHA5 gave rise to two possible reasons for the failure of experiments designed to study partitioning of chromosomes in spherical cells, described in section 3.2.1.

[i] The recombination events causing segregation of alleles in SHA5 and its derivatives, could also occur between heterozygous chromosomes. That is, *E. coli* could use general homologous recombination for homozygotisation of large homologous regions of DNA containing small mismatches. If this happened very quickly, heterozygotes might be too transient to be detected.

[ii] In-depth analysis of the results displayed in table 3.2.11 uncovered a serious anomaly. The cells on the plates were spherical and so previous data indicated that they all had at least four chromosomes (Donachie and Begg, 1989). The irradiated cells were being induced to cause the two alleles to segregate at a higher frequency than the original SHA8 strain.

This extra segregation must have happened on the plate because irradiation occurred after the cells were streaked out. If one chromosome segregated its two markers (either way) it immediately produced the situation desired in the experiments in section 3.2.1. That is, the cell contained two different types of chromosome. [Indeed this is true for the production of a segregant chromosome even without UV induction.] This cell went on to produce a colony which was screened to give the data in table 3.2.11. Even if the two types of chromosome in this colony segregated from one another so that no cell in the colony contained both, the colony should have contained a mixed population of cells. Table 3.2.11 shows that only 9% (4/46) of all segregants detected in the experiment were in mixed colonies. When all the segregation is happening *in situ* on the plate one would expect mixed colonies to be predominant over pure segregants. Two possible explanations for this anomaly are, **A:** once one chromosome has segregated to homozygosity for one marker it is highly unlikely that any of the remaining chromosomes will segregate in the other 'direction'; **B:** spherical cells in colonies are not polyploid. Hence a mixed colony can only be produced by reciprocal segregation in a cell which has two copies of the appropriate region. This would be during the part of the cell cycle from when that region is replicated to the time when the septum is formed.

3.2.3 Final experiments on partitioning in spherical cells

Further experiments to investigate partitioning in spherical cells were designed, taking into account the possible problems discussed at the end of the previous section.

Transductions into temperature sensitive recA mutants. The possibility was tested, that the system which is responsible for the segregation occurring in SHA5 and its derivatives, has the same effect on heterozygous chromosomes.

It has already been shown that the recombination responsible for segregation does not occur in a *recA* mutant (*3.2.2*). Unfortunately

the experiments cannot be done in a *recA* strain because the transduction process relies on homologous recombination. It was proposed therefore to make a *recA*^{ts} version of the strain KJB24 so that transduction experiments could be carried out at the permissive temperature (*recA*$^{+}$) and then expression and plating out could be done at the non-permissive temperature (*recA*$^{-}$). Theoretically, this would allow the formation of a chromosomal heterozygote but not allow the homozygotisation to take place. Strain LM5 (KJB24 recA^{ts}) was constructed by co-transduction of the *recA*^{ts} allele from N1715 with a proximal Tn*10* marker. Strain LM6 was constructed by transduction (at 30°C) of LM5 to *leu*::Tn*9*; Cm^{R}. Strains LM5 and LM6 were used in transduction experiments similar to those in section 3.2.1, that is, LM5 was transduced to *leu*::Tn*9* and LM6 was transduced to *leu*$^{+}$ and both were plated on M9Cm plates. The transduction was carried out slightly differently to utilise the *recA*^{ts} mutation; incubation of cells with lysate was done at 30°C, incubation for expression of the Cm^{R} marker was at 42°C for 1 h, the cells were plated out on M9Cm at 42°C (*2.4.6*). The growth rate of the spherical cells was such that an absolute maximum of 2 divisions could occur during the expression time so it was predicted that this experiment would detect heterozygotes if partitioning was either mitotic or random (providing the presence of *recA*^{ts} made a difference).

The results of these two experiments were that neither produced any Cm^{R}, *leu*$^{+}$ transductants, however the failure of this experiment (and the other transduction experiments in section 3.2.1) was inevitable as shown by the subsequent results described below.

Spherical and rod-shaped cells have similar size distributions in stationary phase. The suggestion that spherical cells may not be polyploid in all phases of growth (*3.2.2*) was investigated by measuring the volume of the cells from colonies and in stationary phase cultures and comparing them with similar measurements for W3110. The volume was measured because of the correlation between the large size and high DNA content of the spherical cells in log phase cultures (Donachie and Begg, 1989) and indeed the correlation between DNA content and size in wild-type *E. coli*

(Donachie, 1968). Cells from a single colony of KJB24 and a single colony of W3110 were resuspended in Bacterial buffer and then estimated using a Coulter Channelyser (*2.3.4*). Cultures of KJB24 and W3110 were grown overnight in various liquid media (LB, NB, M9 containing Cαα, M9). These cultures were also analysed for cell volume as above. The results are displayed in Figure 3.2.1 along with data for log phase cells from results in the following section.

The results showed that spherical cells (KJB24) were consistently bigger than rod-shaped cells (W3110) even in stationary phase but this was by a factor of 5–33% compared with approximately 400% in log phase cultures. It seems possible therefore that the spherical cells transduced in previous experiments (which were from standing overnight culture in LB (*2.4.6*)) did not contain multiple chromosomes because they were in or near to stationary phase. It also provides a possible explanation for the absence of mixed colonies in the UV induced segregation experiments described in section 3.2.3.

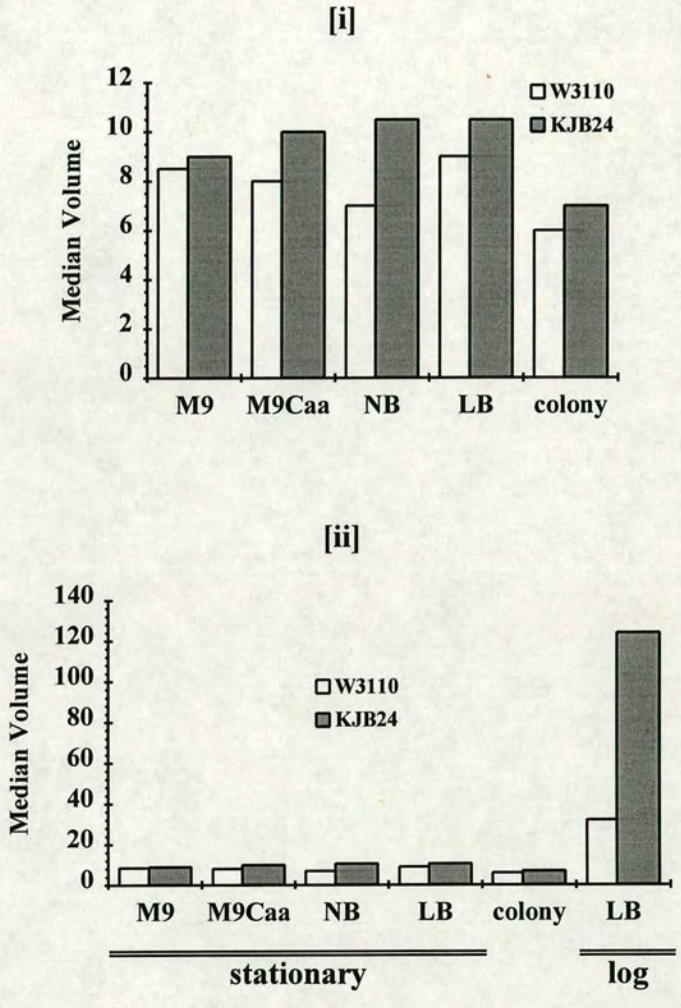
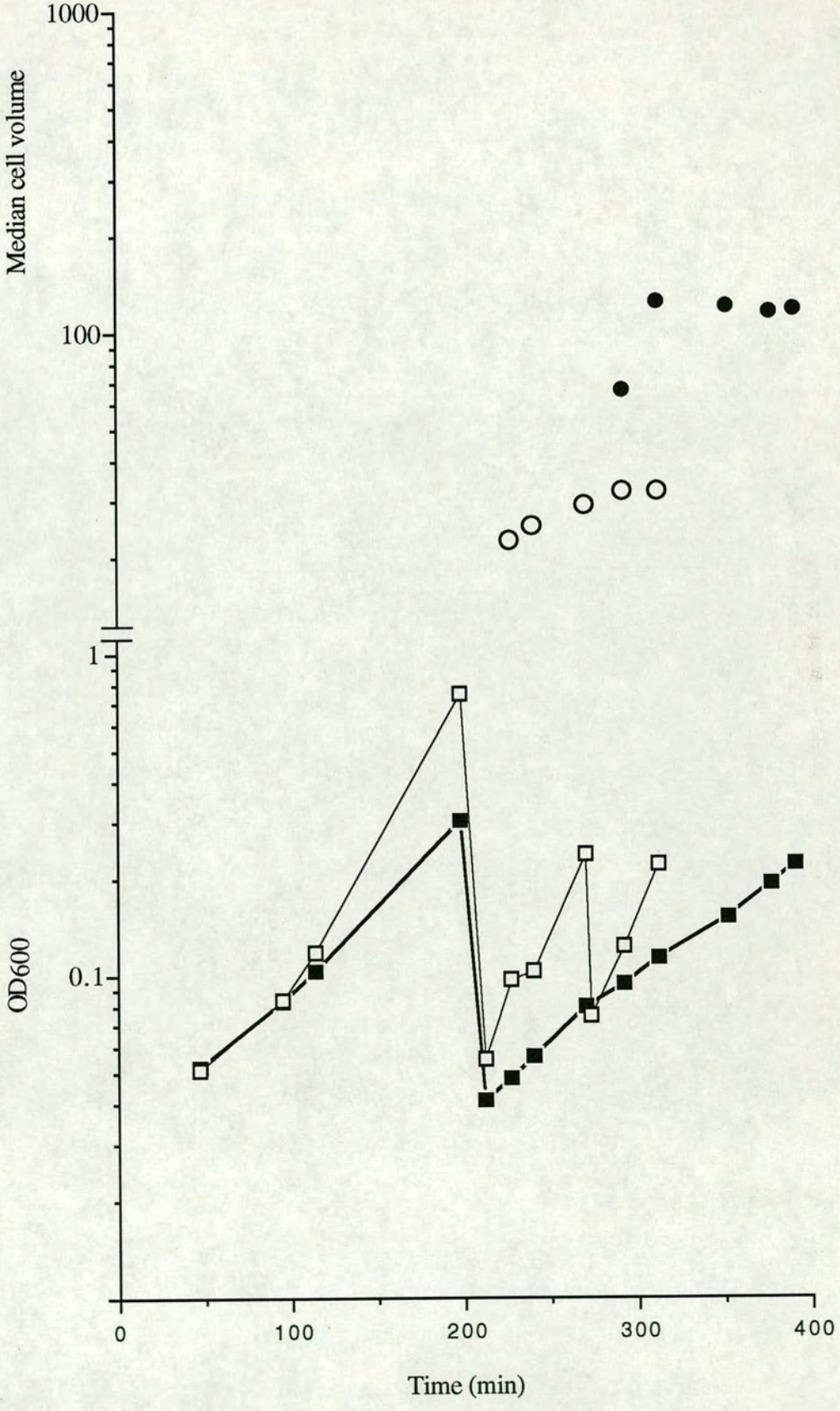


Figure 3.2.1 *Size analysis of spherical and rod-shaped cells.* [i] Comparison of KJB24 and W3110 in stationary phase and in colonies. [ii] Comparison of the same data with values for log phase cultures. The y-axis values are the median cell volume, calculated using the Coulter Channelyser (*2.3.4*). The x-axis shows the different conditions under which the cells were grown.

Transduction of cells in log phase. Following on from the results showing that spherical cells were approximately the same size as rod-shaped cells in stationary phase, transduction experiments, similar to those described in section 3.2.1, were carried out on recipient cells which were in log phase. The spherical cells would therefore have the large number of chromosomes essential for the original premise of these experiments. Cultures of KJB24 and W3110 were grown in LB with vigorous shaking and their OD_{600} was monitored. When the cultures reached an OD_{600} of approximately 0.2, 1.5 ml of culture was pelleted in a microfuge, resuspended in 100 µl of LB containing Calcium and used as recipient cells as described (*2.4.6*). Both cultures were transduced to *leu*::Tn*10* and plated onto both LBT and M9T plates. A typical growth curve of the two strains is depicted in Figure 3.2.2 along with their median volumes (previously shown in Figure 3.2.1).

Figure 3.2.2 *Comparison of spherical and rod-shaped cells.* The graph displays the OD_{600} of W3110 (□) and KJB24 (■) and the median cell-volume of W3110 (○) and KJB24 (●). Overnight cultures were diluted into fresh LB at 0 min and then grown at 37°C.

The two features of note from the growth curve are [i] that the spherical cells (KJB24) are approximately 4x bigger (124/32) than the rod-shaped cells (W3110) and [ii] the spherical cells have a slower growth rate than the rod-shaped cells. This is probably a consequence of having accumulated a slow growth mutation which allows the spherical cells to grow in rich medium (*Discussion, 3.3.1*). A prediction from this observation is that comparisons of cultures of these two strains growing at the same rate, the spherical cells would be even bigger than the rod-shaped cells. In this work however, the two strains were always grown in comparative conditions so the figure of a factor of four has been used throughout.


1000
100
1
0.1
Median cell volume
OD600
0
100
200
300
400
Time (min)

For both strains, the number of colonies on LBT plates was approximately 2x the number on the M9T plates. Colonies patched from all plates gave identical results, that is, only normal transductants were detected whether the recipients were rod-shaped or spherical and whether there was double or single selection. The colonies on the M9T plates were therefore spuriously *leu*$^+$. *Vide supra* (*3.2.1*). The fact that results were identical for both cell shapes is now significant. The same experiment was done comparing log phase and stationary phase spherical cells with similar results.

The conclusion must be that it is impossible to isolate chromosomal heterozygotes from polyploid spherical cells. It is also virtually impossible to find evidence for the *unavoidable* initial presence of heterozygotes immediately after transduction. The implications of this conclusion on the nature of partitioning in spherical cells (and ultimately on rod-shaped cells) are discussed in section 3.3.

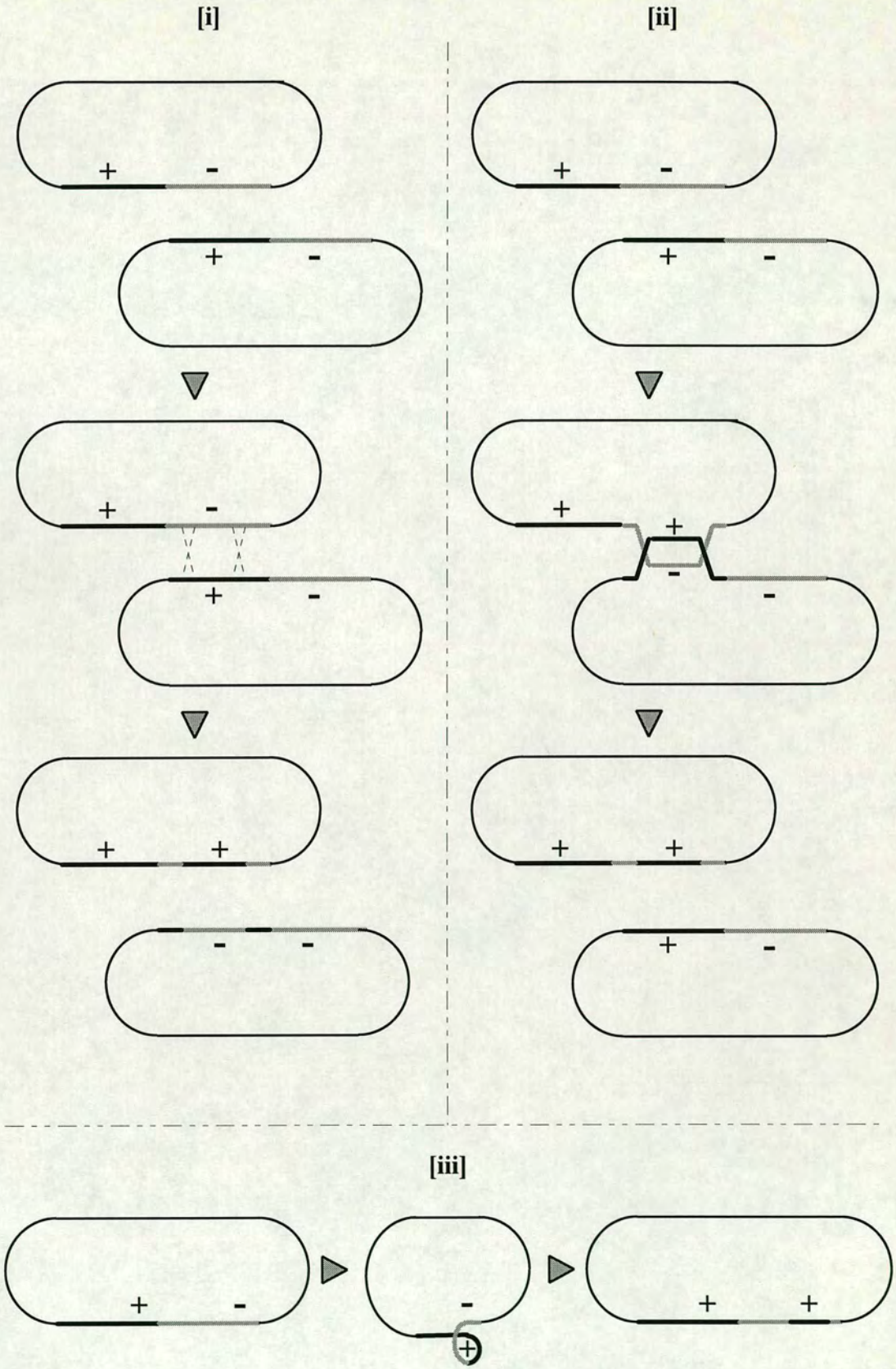
3.3 Discussion

3.3.1 Partial diploid strains

Unbeknownst to this laboratory, the spontaneous production of partial diploid strains had been reported before, both in *Salmonella typhimurium* (Anderson and Roth, 1981) and in *Escherichia coli* (Lin *et al.*, 1984). The large duplications, resulting from recombination between repetitive sequences in the chromosome (*rrn* genes, Anderson and Roth, 1981 or *rhs* sequences, Lin *et al.*, 1984) their unstable nature and the *recA* dependence of this instability had all been previously noted. The instability was reported to be due to reversion back to haploidy, presumably by the reverse of the process which caused the duplications. The fact that 'partial-diploid' strains occasionally stabilise was also reported; one group attributed this to transposition of one of the detectable markers (Lin *et al.*, 1984). In *S. typhimurium* it was shown that approximately 0.03% of a population of cells contain a duplication at the 2' region but that some loci near to *rrn* sequences could be duplicated in 3% of cells (Anderson and Roth, 1981).

A new feature uncovered by the study of SHA5 was the maintenance of diploidy subsequent to segregation from heterozygosity. This phenomenon could have arisen from three possible events, [i] an intermolecular reciprocal recombination event between opposite copies of the duplicated region on different chromosomes in the same cell, [ii] an intermolecular gene-conversion event between opposite copies of the duplicated region on different chromosomes in the same cell or [iii] an intramolecular gene-conversion event between the two duplicated segments on the same chromosome. This is illustrated in Figure 3.3.1.

Figure 3.3.1 *Possible pathways to producing homozygous 'partial-diploids'.* Thick black and grey lines indicate the two versions of the duplicated region. Thin black lines indicate the rest of the chromosome. Dotted lines indicate homologous recombination. The + and - signs indicate the two detectable alleles. [i] One interchromosomal reciprocal recombination event leaves two 'opposite' homozygous, 'partial-diploid' chromosomes. [ii] One interchromosomal gene-conversion event leaves a homozygous, 'partial-diploid' chromosome and an unchanged heterozygous chromosome. [iii] One intrachromosomal gene-conversion event leaves a homozygous, 'partial-diploid' chromosome. If [i] happens in a polyploid cell, the cell will then contain three types of chromosome (in terms of the detectable phenotypes, ++, --, +-). Similarly, [ii] and [iii] both produce a cell containing two types of chromosomes. In all cases, a polyploid cell would have to undergo segregation of the chromosomes by partitioning or further recombination events, in order to produce a pure segregant. All three processes could theoretically happen in a cell with a single chromosome; [i] and [ii], between two arms of a chromosome which has replicated past the position of the duplication. Therefore, unfortunately, it is not possible to distinguish between these three possibilities using either the results presented here or previously published results.


[i]
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The results showing that SHA5 pSU66 was much more unstable than SHA5 and that most of its segregants were haploid suggests that chromosomal duplications are more stable in the spherical cells. This would allow the production of homozygous 'partial-diploids' to be detected more easily in spherical cells, however they *were* detected, at a much lower frequency, in the rod-shaped SHA6 pSU66 transformants (*3.2.2*). That the strains used by other workers were rod-shaped might explain why homozygous 'partial-diploids' were not reported by them.

It is difficult to explain why the duplications are more stable in spherical cells than rod-shaped cells. Two possible suggestions are: [i] The large size of the spherical cells allows them to accommodate more DNA in terms of larger chromosomes (as well as more chromosomes). This seems an unlikely explanation because of the result showing that spherical and rod-shaped cells have essentially the same volume in stationary phase, however the recombination events which cause the instability could happen predominantly when the cells are actively growing; [ii] Null mutations in the *rodA* gene are considered to be lethal by some workers (Vinella *et al.*, 1990), however other workers have found to the contrary and indeed such mutants seem to grow well on minimal medium (Spratt, 1980, KJ Begg, personal communication). Their improved survival in minimal medium is probably due to an increase in the intracellular pool of guanosine 5'-diphosphate 3'-diphosphate (ppGpp) associated with the slow growth, which is known to help some spherical mutants to survive (Vinella *et al.*, 1993). The growth curve shown in Figure 3.2.2 reveals that KJB24 has a reduced growth rate compared with its parental strain W3110. This reduction in growth rate allows KJB24 to grow in rich medium, contrary to the literature. During this work (results not presented in thesis) I have shown that if KJB24 is transduced back to *rodA*$^+$ it still has a reduced growth rate. This indicates a genetic change which is likely to be one of the reported suppressers of spherical lethality which cause an increase in the pool of ppGpp and/or cause a decrease in the growth rate (Ogura *et al.*, 1989, Vinella *et al.*, 1993). The duplication of up to 12' of the chromosome would inevitably lead to chromosome replication taking longer than normal. Therefore it is possible that the reason for the extra stability

of duplications in spherical cells is that their slow growth rate could allow more time for the large chromosome to be replicated. This is not a wholly satisfactory explanation because the pSU66 transformants of SHA5 and SHA6 will also inherit the slow growth mutation.

The real reason may be a combination of both large size through most of the cell cycle and slow growth rate.

3.3.2 Partitioning in spherical cells

Ultimately, only the final experiment in section 3.2.3 can be used to draw conclusions about the partitioning process. It should perhaps not have been surprising that the results expected for mitotic partitioning were not observed, since no difficulty in the genetic transformation of spherical strains of *E. coli* has been reported previously. Random partitioning, however, could quite easily have been overlooked because routine transductions do not involve selecting for alleles which are to be removed from the cell. Also if a standing overnight culture of recipient cells is used then the cells will be nearing stationary phase anyway. The fact that a chromosomal heterozygote cannot be detected even when multichromosomal, log phase recipient cells are used suggests that partitioning is happening in a very specific way. It only appears to be compatible with the model depicted in Figure 3.1.4 [i], that is, non-random, perimeter-mediated partitioning. A more detailed model for the partitioning of chromosomes in spheres is discussed below.

The hierarchical model of chromosome partitioning and segregation. A model for partitioning in spherical cells must take into account the following:

[i] Spherical cells are at least four times the volume of equivalent rod-shaped cells in log phase but are the same size in stationary phase.

[ii] Spherical cells have approximately four times as many chromosomes as equivalent rod-shaped cells in log phase but the same amount in stationary phase. This is probably a direct result of [i] (see Donachie, 1968).

[iii] When spherical cells are observed growing in micro-colonies on agar, the plane of one division appears to be perpendicular to the plane of the previous division. Also, septation is initiated asymmetrically, from the side of the cell nearest its sister (Iwaya *et al.*, 1978, KJ Begg unpublished); see Figure 3.3.2.

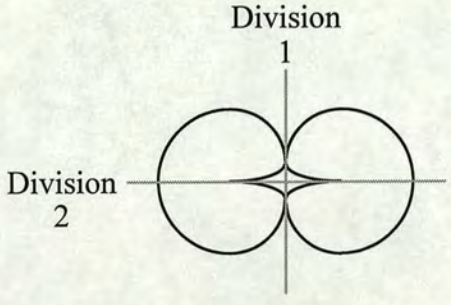


Figure 3.3.2 *Alternate planes of division and asymmetric initiation of septation in spherical E. coli cells.*

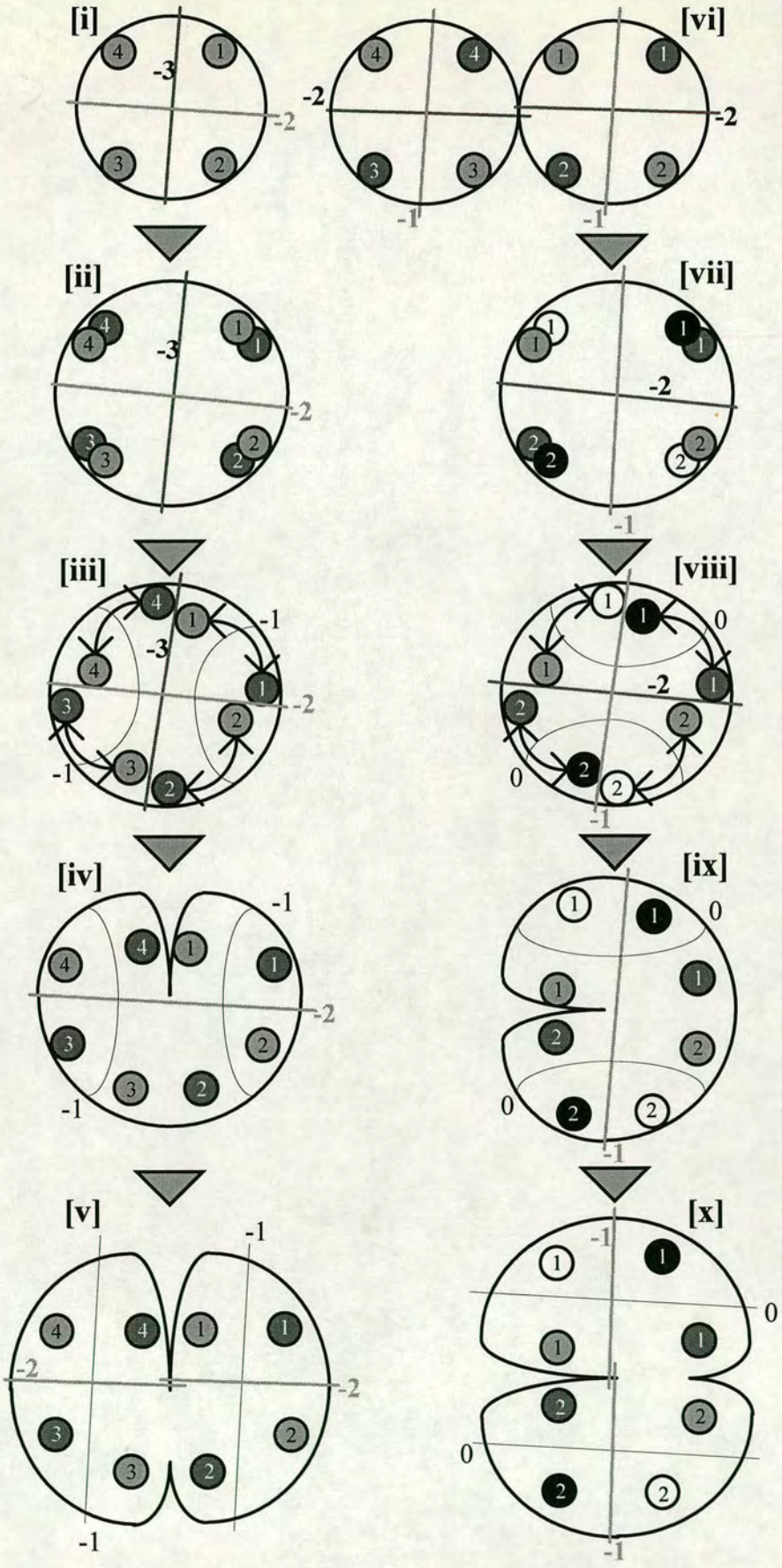
[iv] Chromosomal heterozygosity cannot be maintained or even transiently detected in multichromosomal spherical cells.

It was stated in section 3.1.1 that the segregation characteristics of chromosomes partitioned in a perimeter-mediated, non-random fashion would depend on the relationship between partitioning and placement of the septum. As stated above this is the only remaining mode of partitioning which could fit with the data described in the preceding sections. The model described fully in Figure 3.3.3 defines the relationship between partitioning and septation which is necessary to explain the total failure to detect chromosomal heterozygosity in spherical cells.

Figure 3.3.3 *The hierarchical model of chromosome partitioning and segregation.* Arrowed lines indicate the result of the action of the partitioning apparatus, which is perimeter-mediated and non-random in this model; see Figure 3.1.4. Small circles indicate separate chromosomes, numbers therein distinguishing between individuals in the initial cell. Large outlines indicate log phase spherical cells. Lines crossing cells indicate the presence of predetermined division axes.

The hierarchical model for chromosome partitioning and segregation proposes that each partitioning event absolutely determines a future division site or axis. This axis is laid down between the two chromosomes and so ensures their separation into two different cells when the axis is used. A multichromosomal spherical cell is considered to be delayed in division, such that associated partition and division events take place two generations apart. This does not mean that division is slow but that, on exit from stationary phase, they effectively grow without dividing for two generations (thus explaining *how* spherical cells become large, although not *why*). Division then starts at the oldest axis laid down by previous partition events. Partitioning, by laying down an obligate division site or axis between two chromosomes, ensures their segregation two generations hence. Chromosomal heterozygotes are therefore maintained for a maximum of two generations. On entry into stationary phase a cell would divide at the remaining two axes, without growth, to form four unichromosomal grand-daughters. The model is hierarchical in two ways. The cell uses the oldest division axis present, so that it divides in half; the relationship between partitioning and division ensures that each chromosome is destined to be the sole determinant of a cell line.

[i] A new-born spherical cell containing four chromosomes inherits two predetermined axes for division. (The axes are numbered such that the last partitioning event illustrated in this figure is 0, the penultimate is -1 and the two which happened 'before' the figure are -2 and -3.) **[ii]** Replicated chromosomes are about to be partitioned. Shading differentiates identical sister chromosomes. **[iii]** Partitioning occurs and new division axes, -1, are created between the chromosomes. This could also be considered as four potential-division-sites (PDS, Donachie and Begg, 1970), one between each pair of partitioned chromosomes, which will subsequently mature to direct the formation of a septum. **[iv]** Division starts along the oldest axis, -3. **[v]** As division continues, the axis determined by partitioning event -2 is split. Here, the alternative idea of one PDS being inherited by each cell is more attractive. **[vi]** The cell has completed division. Each daughter cell inherits two predetermined division axes. Subsequent illustrations are of the right hand cell only. **[vii]** As for [ii]. **[viii]** Partitioning occurs and new division axes (or four PDS), 0, are created. **[ix]** Division starts along the oldest axes, -2. **[x]** Division continues as in [v]. In the next round of division, axes -1 will be used two generations after they were created, in [iii]. It can be seen that after division -2 the four original chromosomes in [i] will be completely separated from one another. Thus even a chromosomal 'tetrazygote' is homozygotised by two divisions, and each chromosome produces its own clone of cells.


[i]
[ii]
[iii]
[iv]
[v]
[vi]
[vii]
[viii]
[ix]
[x]

It must be stressed that the model in Figure 3.3.3 does not identify the structures involved in the various processes described, however many structural aspects of these processes are known and others can be speculated upon.

Division machinery. The division apparatus has been extensively studied and is discussed in detail in Chapter 1. It is reasonable to assume that known cell division proteins such as FtsZ, FtsI, FtsQ, FtsA and others are involved in the formation of the septum of spherical cells in the same way as in rod-shaped cells (Donachie and Begg, 1990). The time taken for a septum to form in spherical cells is comparable to that in rod-shaped cells (KJ Begg unpublished) and so cannot account for their large size. The only difference between the two appears to be the initially asymmetric nature of the septum in spherical cells. It is therefore possible that the production or shape of the FtsZ ring (Bi and Lutkenhaus, 1991) may be slightly different in spherical cells. One could draw a connection between this and the idea that lethality of spherical mutants is due to insufficient FtsZ protein for their large circumference. Vinella *et al.* (1993) showed that introduction of a plasmid containing *ftsZ, A* and *Q* genes (pZAQ) could suppress the lethality of a *pbpA* deletion, however; [i] spherical cells have a concomitantly increased copy number of the *ftsZ* gene due to being multichromosomal, also SHA6 which has double the number of copies of the *ftsZ* gene again, appears not to divide any differently to KJB24; [ii] the pZAQ plasmid, used by Vinella *et al.* has been observed to slow the growth rate of host cells (KJ Begg unpublished) and slow growth with its resulting increase in the ppGpp pool, is known to help the growth of spherical mutants; [iii] introduction of a different FtsZ overproducing plasmid, pSUZ, into spherical cells has conversely been shown to be detrimental, leading eventually to lysis (KJ Begg unpublished).

Division axes. There are candidates for the proposed division axes laid down by partition events. For example, the FtsZ ring and/or whatever guides its polymerisation, or a structure like the periseptal annulus observed by Cook *et al.* (1987) would both provide a circumferential axis; the filaments detected by overproduction of CafA

(Okada *et al.*, 1994) would provide a cytoplasmic axis. (Whether any of these structures actually form in spherical cells is not known, but regular septa *do* form and it is not unreasonable to infer the formation of other structures potentially involved in cell division). The alternative idea is that a PDS would form at a site on the cell perimeter and eventually mature into, or direct the formation of, a structure like those mentioned above. It could be this period of maturation, around the cells circumference, which causes the initial delay in division in spherical cells, leading to their large size in log phase. This could also account for asymmetric division if the septum initiated from the point where the structure is completed (opposite the site of the PDS).

Partitioning apparatus. The hierarchical model proposes a connection between the structures discussed above and the partitioning apparatus. Present knowledge of the latter is discussed in Chapter 1. It is interesting however, that the only two proteins at present which are implicated in the partitioning process *per se* are TolC, an outer membrane protein, and MukB, a large cytoplasmic protein which has strong similarities to eukaryotic force generating proteins. The involvement of TolC would fit with a perimeter-mediated model for partitioning and it would also be a potential component of the PDS. MukB is proposed to be involved in the physical moving apart of chromosomes, an integral part of this model.

Chromosome positioning. The spatial arrangement of chromosomes in the cell would be a consequence of the direction and distance of movement of the partitioning process. It would be a three dimensional arrangement, unlike Figure 3.3.3, but the figure reflects that it would have to be regular and associated with the cell surface. The actual arrangement of chromosomal DNA within spherical cells *in vivo* is unknown at present, although electron-micrograph sections of spherical mutants show the DNA arranged as a shell around the cell surface (Normark, 1969, Iwaya *et al.*, 1978).

General morphological considerations. The alternate planes of division shown by spherical *E. coli* strains would be a consequence of the

hierarchical use of potential division axes, which are in turn proposed to be a direct result of the positions of the chromosomes and the orientation of the partitioning process (Figure 3.3.3). It may be useful to think of a spherical *E. coli* cell with four chromosomes as a similarly endowed rod-shaped filament which can divide once per generation time. This is a situation which cannot really happen *in vivo* but, such short filaments produced by a reversible division block often show a hierarchy of division when the block to division is removed, with the oldest potential division site being used first (KJ Begg, WD Donachie, personal communication) but not always (see Figure 7 in Donachie *et al.*, 1984). Figure 3.3.4 illustrates this situation where a filament is returned to division which, because it produces four unichromosomal cells from one multichromosomal cell, is comparable to the situation when a spherical cell returns to stationary phase.

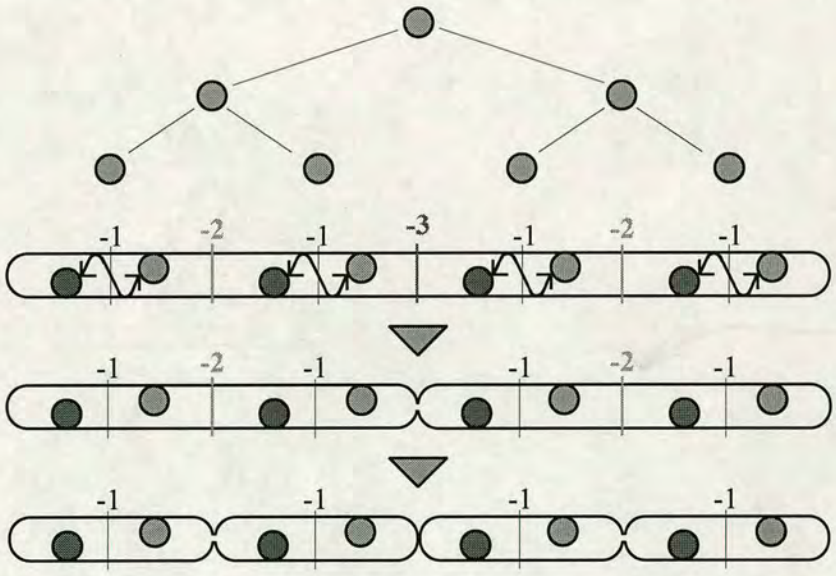


Figure 3.3.4 *Hierarchical use of potential division sites in short E. coli filaments.* All features of the diagram are as in previous figures. This shows hierarchical partitioning and division in action in a filament. Division -3 separates four chromosomes which are direct descendants of one chromosome, from another four which are direct descendants of its sister. Hence when these two sisters were partitioned two generations ago they were destined to end up in separate cells.

In a filament, the inevitable segregation of heterozygotes is an obvious consequence of division. That it happens in spherical cells, indicates that a similar relationship between partitioning and division exists (which is not as obvious because of the shape). This supports the idea of a cellular axis determined by an intrinsic property of the cell surface which is not lost in spherical mutants. In the case of a filament, successive divisions are in the same plane, however if one imagined it curled up into a ball one could probably find a position in which perpendicular divisions would be inevitable. For example Figure 3.3.5.

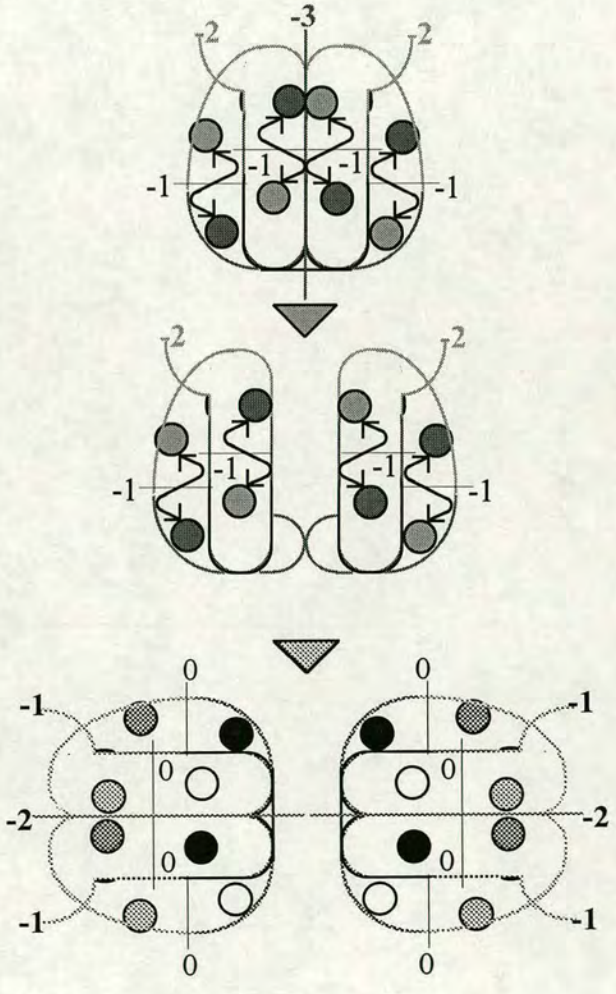


Figure 3.3.5 *Imagine a sphere as a rolled up filament.* This is an amalgam of Figures 3.3.3 and 3.3.4 in order to suggest that a dividing sphere could be thought of as a curled up filament, at least in terms of imagining an axis throughout the cell along which chromosomes must be partitioned. That is, this axis need not be straight or circular.

Conclusions. The ultimate aim of these experiments was defined in the introduction to this chapter; that was, to find out *what* happens to chromosomes during the partitioning process, rather than *how* it happens. The former is now clear; a partitioning event absolutely determines that the two chromosomes involved will be separated into different cells by a division between them. This ultimately means that any one chromosome will always give rise to a pure clonal cell-line. This knowledge will be helpful for a proper understanding of the partitioning and division apparatus of *E. coli*. Many coccal bacterial species show alternating planes of cell-division (for example, *Neisseria*, *Lampropedia*, *Sarcina*) and others are known to be multichromosomal, showing active homozygotisation of mutations (*Azotobacter*, Phadnis *et al.*, 1988, Moldanao *et al.*, 1992; *Micrococcus*, Hansen, 1978, Tigari and Moseley, 1980, Masters *et al.*, 1991). Experiments described by Moldanao *et al.* (1992), showing that recessive markers introduced into *Azotobacter vinelandii* appear after approximately 7 generations, were thought to disprove previous suggestions that the species contained 40–80 chromosomes. It is significant that in a bacterium containing 40–80 chromosomes, with a hierarchical partitioning system, one would expect exactly this result.

It appears therefore that these experiments have uncovered an innate property of partitioning and division in *E. coli* which is common or even a general rule in prokaryotes. This is normally concealed by the actions of the RodA and PBP2 proteins, which make *E. coli* rod-shaped and monochromosomal. Hierarchical chromosome segregation is totally different from the mitotic segregation of eukaryotes and is therefore yet another fundamental point of distinction between the two Kingdoms.

CHAPTER 4

SEQUENCE ANALYSIS OF THE FIFTEEN MINUTE REGION

4.1 Introduction

The *mrd* (*m*urein *r*egion *d*)-cluster of genes, situated at approximately 14.4' on the *E. coli* chromosome contains genes which have major functions in peptidoglycan synthesis and the maintenance of cell shape; see Chapter 1. The operon is situated in a larger region, from 14.3'–14.7' (the fifteen minute region) which is incompletely sequenced and contains at least four genes of unknown function. The aim of this work was to sequence the gap in the fifteen minute region and subject the data for the whole region to thorough computer analysis: the ultimate aim being to provide insight into the functions of some of the uncharacterised genes and to investigate the idea that the *mrd*-cluster constitutes an operon.

4.1.1 The genes of the 15' region

Figure 4.1.1 shows the arrangement of genes in the fifteen minute region, taken from previously published sequence data. A summary of the functions of these genes is presented in Table 4.1.1; see Chapter 1.

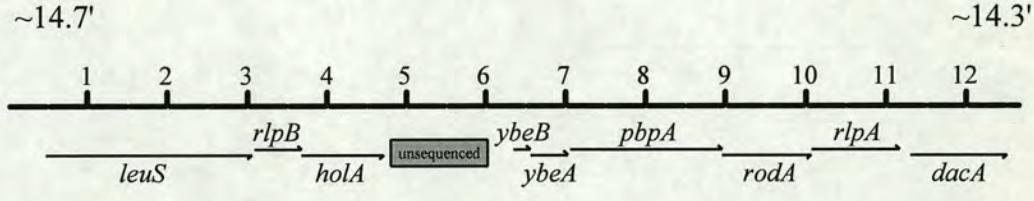


Figure 4.1.1 *Map of the fifteen minute region of the E. coli chromosome.* The upper line indicates the scale in kilo-base pairs (kb). Thin lines with arrow-heads indicate coding regions of genes and their direction of transcription. The shaded box represents the unsequenced portion. Sequenced non-coding regions or overlaps (indicated by a negative sign) are as follows: ⇨*leuS* = 490 base pairs (bp); *leuS* ⇨*rlpB* = 17 bp; *rlpB* ⇨*holA* = -1 bp; *holA* ⇨unsequenced = 99 bp; unsequenced ⇨*ybeB* = 323 bp; *ybeB* ⇨*ybeA* = 6 bp; *ybeA* ⇨*pbpA* = 33 bp; *pbpA* ⇨*rodA* = 5 bp; *rodA* ⇨*rlpA* = 13 bp; *rlpA* ⇨*dacA* = 141 bp; *dacA*⇨ = 146 bp. Unsequenced DNA lies at either end of the region.

Table 4.1.1 The genes of the fifteen minute region of the *E. coli* chromosome

Gene	Description	Reference
leuS	Gene encoding leucyl-transfer ribonucleic acid (tRNA) synthetase.	Low *et al.*, 1971.
rlpB	Gene encoding rare lipoprotein of unknown function.	Takahase *et al.*, 1987.
holA	Gene encoding δ-subunit of DNA polymerase III.	Dong *et al.*, 1993.
ybeB	Gene encoding protein of unknown function.	Asoh *et al.*, 1986.
ybeA	Gene encoding protein of unknown function.	Asoh *et al.*, 1986.
pbpA	Gene encoding penicillin-binding protein (PBP) 2. Peptidoglycan synthesis activity responsible for maintenance of cell shape.	Asoh *et al.*, 1986. Ishino *et al.*, 1986.
rodA	Gene encoding accessory protein to PBP2, also responsible for maintenance of cell shape.	Matsuzawa *et al.*, 1989. Ishino *et al.*, 1986.
rlpA	Gene encoding rare lipoprotein of unknown function.	Takahase *et al.*, 1987.
dacA	Gene encoding PBP5. D-alanine carboxypeptidase activity.	Broome-Smith *et al.*, 1988.

4.2 Results

4.2.1 Sequencing of the gap in the fifteen minute region

It has been shown that *ybeB*, *ybeA*, *pbpA*, *rodA* and *rlpA* probably constitute a single transcriptional unit (Matsuzawa *et al.*, 1989). This will be called the *mrd*-operon. It was noticed that those genes to the left (in Figure 4.1.1) of the unsequenced portion had no obvious connection to the genes of the *mrd*-operon (to the right) and therefore it was reasonable to assume that the start of the *mrd*-operon was situated somewhere between the end of *holA* and the start of *ybeB*. This portion was therefore sequenced.

Sequencing was carried out as described in Chapter 2 (*2.2.15*) using M13 no.6 and M13 no.7 for single stranded templates and pADD2 for double stranded template. Oligonucleotide primers were designed to anneal to the template approximately 30 bases upstream of previous sequence so that overlaps could be confirmed. The sequence was read on both strands for its entire length and was continued until overlaps with published sequence data were detected. Sequencing strategy and results are presented in Figures 4.2.1 and 4.2.2 respectively.

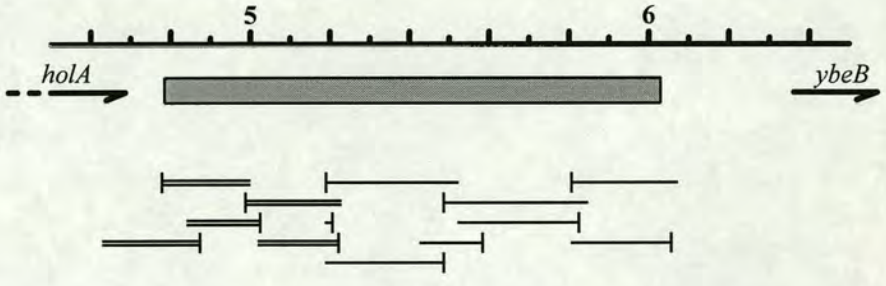


Figure 4.2.1 *Sequencing strategy.* This is an enlargement of the region between 4.5 and 6.5 kb. from Figure 4.1.1, with the previously unsequenced region indicated by the shaded box. The end of *holA* and the whole of *ybeB* are illustrated. Small divisions in the scale (above) are 100 bp apart. Horizontal lines below the map indicate sequences read from various primers with vertical lines indicating where the reading starts. Single and double horizontal lines indicate single and double-stranded sequencing respectively.

Figure 4.2.2 *Sequence of the gap in the fifteen minute region.* The non-coding strand is shown and so RNA sequence is as written (substituting 'U's for 'T's). Dots above the sequence are every 10 base pairs (bp). bp are numbered on the left starting one bp after the stop codon of *holA*. Therefore the first 99 bp is previously published sequence however a number of errors were corrected in this region. Also included is the 323 bp of previously published sequence upstream of *ybeB*. Novel sequence is therefore bp 100–1322. Two new open reading frames, discussed below, are indicated by bold type with a horizontal line above. Start codons are indicated by >>> and stop codons are indicated by ***.

4.2.2 Analysis of novel sequence data

Open reading frames. The novel sequence was analysed using the GCG7 programs on ERCVAX (Genetics Computer Group, 1991). Using the "translate" command, the sequence was translated in all six reading frames. This uncovered two obvious open reading frames in the new sequence, both of which were in the same orientation as all the other genes in the region; see Figure 4.2.2. The first open reading frame, *orfUU*, started with an ATG codon at bp 2 and finished with a TGA codon at bp 641 of Figure 4.2.2 and was equivalent to the start of an open reading frame noticed by Dong *et al.* (1993). This open reading frame started one bp from the stop codon of *holA* and so was +1 in frame from *holA*. A few errors in this part of the published sequence were corrected but there were none in the small overlap sequenced into *holA* itself. The second open reading frame, *orfU*, started with one of the ATG codons at bp 664 and 667 and finished with a TGA codon at bp 1276; see Figure 4.2.2. *orfU* was -1 in frame with respect to *orfUU* with a gap of 20 or 23 bp between the two, depending on which start codon was used. This left 367 bp to the start of *ybeB*. Figure 4.2.3 shows the new map of the fifteen minute region with *orfUU* and *orfU* illustrated.

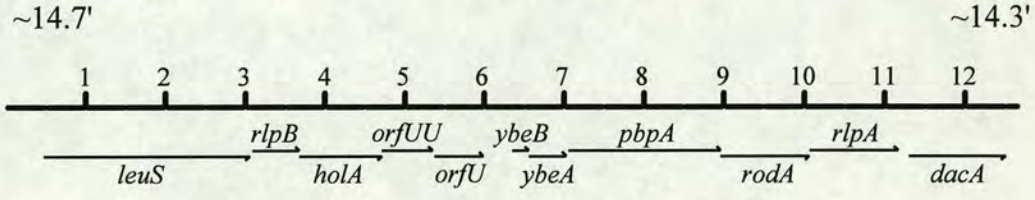


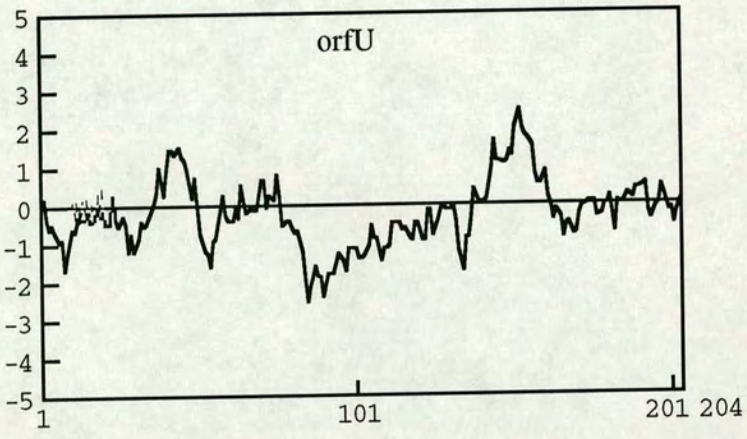
Figure 4.2.3 *Complete map of the fifteen minute region of the E. coli chromosome.* The upper line indicates the scale in kilobase pairs (kb). Thin lines with arrow-heads indicate coding regions of genes and their direction of transcription.

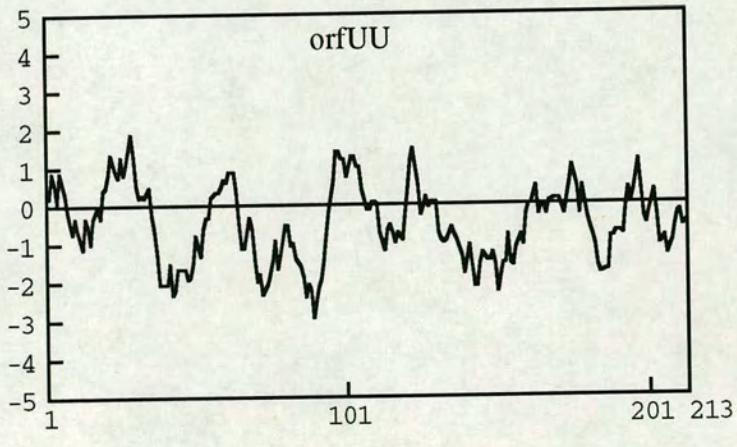
At 639 and 612 bp respectively (longer than both *ybeB* and *ybeA*) *orfUU* and *orfU* were considered to be long enough to be genes and so were further investigated.

Promoter and Shine Dalgarno sequences. It is particularly important to know the positions of promoters in a region which may contain an operon. Therefore, as is customary when new open reading frames are identified, searches for possible promoter sequences and ribosome binding sites (rbs, Shine and Dalgarno, 1974) were carried out with respect to *orfUU* and *orfU*. This was done by eye, using consensus sequences for promoters described in Hoopes and McLure (1987) and looking for sequence similarity to the 3' end of the 16S ribosomal RNA (16S rRNA) situated appropriately with respect to the start codon, as described in Gold and Stormo (1987). No consensus or near-consensus promoter sequences with reasonable spacing between '-35' and '-10' boxes were found upstream of either gene, suggesting that they might be transcriptionally coupled to upstream genes. Four bases from the ATG codon of *orfUU*, the sequence **5' GacGGT 3'** has four bases complementary to the 16S rRNA sequence (in capitals). The sequence **5' GGAGG 3'** was found two bases upstream of the first ATG codon at the start of *orfU* and five from the second ATG. These would appear to be the rbs for the two new open reading frames. A spacing of two nucleotides between the rbs and the start codon has been shown to be deleterious to initiation of translation (Gold and Stormo, 1987). This suggests that the second ATG of *orfU* is most likely be the start codon.

Basic secondary structure predictions. The protein sequences from *orfUU* and *orfU* were predicted using the "translate" command from the GCG package. *orfUU* encodes a 213 amino acid predicted product (OrfUU), with an isoelectric point of 5.6 and a molecular weight of 24.528 kiloDaltons (kD). *orfU* encodes a 204 amino acid predicted product (OrfU), with an isoelectric point of 6.28 and a molecular weight of 23.440 kD. Simple hydropathy plots were carried out for both the predicted proteins using the algorithm of Kyte and Doolittle (1982). Figure 4.2.4 shows the results of these and the implications are discussed in the accompanying legend.

Figure 4.2.4 *Hydropathy plots of the predicted products of orfUU and orfU.* The hydrophobicity as calculated by the Kyte and Doolittle algorithm is plotted against the length of the proteins. OrfUU appears to be predominantly hydrophilic with two small regions of hydrophobicity. OrfU has a fairly neutral portion followed by a small hydrophobic region, a large hydrophilic region and then a putative hydrophobic membrane spanning segment.


orfU
5
4
3
2
1
0
-1
-2
-3
-4
-5
1
101
201 204


orfUU
5
4
3
2
1
0
-1
-2
-3
-4
-5
1
101
201 213

Database comparisons. The two new open reading frames were compared with various protein and DNA sequence databases in order to find out if they were related to or identical to any known sequences. Two different DNA sequence comparison strategies and four different protein sequence comparison strategies were used: DNA comparisons were carried out with [i] the "Fasta" command in the GCG package, using the EMBL database and [ii] "DIASRCH" developed by the Bio-computing Research Unit at the University of Edinburgh (BRU), using the gen77 database. Protein comparisons were carried out with [i] the "Tfasta" command in the GCG package, using the EMBL database, [ii] "PROSRCH" (BRU), using the swiss26 database, [iii] "Blitz Search" in the EMBL E-Mail Search program, using the Swiss-Prot database and [iv] "Blast Search" in the GenBank Search program, using the National Centre for Biotechnology database. The searches generally detected short regions of similarity (up to about 100 amino acids) and so complete sequences were then aligned using the "GAP" command from the GCG package. The searches were repeated periodically as the databases were updated and the cumulative results are presented below.

orfUU

DNA and protein database searches failed to detect any similarities to the open reading frame or predicted product of *orfUU*. The phrase 'pioneering protein' has been coined for this eventuality. Figure 4.2.5 shows the predicted amino acid sequence of OrfUU.

```
  1  MKSLQALFGG  TFDPVHYGHL  KPVETLANLI  GLTRVTIIPN
 41  NVPPHRPQPE  ANSVQRKHML  ELAIADKPLF  TLDERELKRN
 81  APSYTAQTLK  EWRQEQGPDV  PLAFIIGQDS  LLTFPTWYEY
121  ETILDNAHLI  VCRRPGYPLE  MAQPQYQQWL  EDHLTHNPED
161  LHLQPAGKIY  LAETPWFNIS  ATIIRERLQN  GESCEDLLPE
201  PVLTYINQQG  LYR
```

Figure 4.2.5 *Predicted amino acid sequence of OrfUU.* The amino acids are numbered on the left. Single letter codes are used for amino acids.

orfU

Searches using OrfU as the query sequence revealed significant similarity to a large family of proteins. These included the predicted product of an uncharacterised open reading frame from *E. coli* (Skarstad *et al.*, 1993)[5] , nine different prokaryotic and eukaryotic phosphoglycerate mutase (PGAM) enzymes and five eukaryotic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PF2K) enzymes. Associations between OrfX and PGAMs (K Rudd *per* J Nairn) and between OrfX and bovine PF2K (PF2K-B, Skarstad *et al.*, 1993) were made previously. Also there is an established connection between PGAM and PF2K enzymes. PF2K is a bifunctional enzyme and can be split into two domains purely on the basis of very strong sequence similarities to known single function enzymes (Bazan *et al.*, 1989). The C-terminal domain of PF2K catalyses the fructose-2,6-bisphosphatase reaction and it has been shown to be directly related to PGAMs by primary sequence alignments, secondary structure predictions and biochemical data. In particular, both enzymes have a 'phosphohistidine' motif and a distant histidine residue which are juxtaposed in the folded protein. They act in substrate binding, by the formation of a phosphohistidine intermediate (the enzyme phosphorylated at the histidine residue) during the catalytic reaction. There are other well conserved residues considered to be at, or near, the active site of the enzymes (Bazan *et al.*, 1989, Fothergill-Gilmore and Watson, 1989).

An alignment between the whole of OrfU and *E. coli* PGAM (PGAM-E), which was first in the list of sequences pulled out of the databases and so was supposedly the best match, is presented in Figure 4.2.6. It shows that OrfU has both the phosphohistidine motif and the distant histidine residue, with a background of reasonable sequence similarity.

---

[5] For ease of reference, the uncharacterised open reading frame which was not given a name by its discoverers, (*op cit*) will be called *orfX* and its product OrfX.

E. coli phosphoglycerate mutase x OrfU

```
                .         .         .         .         .
PGAM-E  1 MAVTKLVLVRHGESQWNKENRFTGWYDVDLSEKGVSEAKAAGKLLKEEGY 50
            : :| |:||||.| | :. :.|  ...|..:|:.:|..  .||  .|
  OrfU  1 __MMRLWLIRHGETQANIDGLYSGHAPTPLTARGIEQAQNLHTLL__HGV 46
                .         .         .         .         .
       51 SFDFAYTSVLKRAIHTLWNVLDELDQAWLPVEKSWKLNERHYGALQGLNK 100
          |||:...| |.|| || : ||.: :   |||:   .|||. :|.::
       47 SFDLVLCSELERAQHTARLVLSDRQ___LPVQIIPELNEMFFGDWE____ 89
                .         .         .         .         .
      101 AETAEKYGDEQVKQWRRGFAVTPPELTKDDERYPGHDPRYAKLSEKELPL 150
                    .. :|::       : .|.|.|.:   ::..: .   .
       90 _____________MRHHRDL______MQEDAENYSA___WCNDWQHAIPTN 118
                .         .         .         .         .
      151 TESLALTIDRVIPYWNETILPRMKSGERVIIAAHGNSLRALVKYLDNMSE 200
          .|::.   :|| .:   . |. :.  :.:::..|.. |. |:  | .|..
      119 GEGFQAFSQRVERF__IARLSEFQHYQNILVVSHQGVLSLLIARLIGMPA 166
                .         .         .         .         .
      201 EEILELNIPTGVPLVYEFDENFKPLKRYYLGNADEIAAKAAAVANQGKAK 250
          |.::.:.:. |.  ..:::::.| .|:          :..:| :|.|.
      167 EAMWHFRVDQGCWSAIDINQKFATLR________VLNSRAIGVENA____ 204
```

Figure 4.2.6 *Alignment between PGAM-E and OrfU.* The two protein sequences are aligned one above the other and are labelled on the left. The amino acids of each protein are numbered at the sides. Dots above the sequences are at every 10 amino acids. Vertical lines between the two sequences indicate identical amino acids, double and single dots indicating conserved and semi-conserved substitutions, respectively. Horizontal lines within sequences indicate gaps inserted to optimise the alignment. The phosphohistidine motif (residues 8–17 of PGAM-E) and the important histidine (residue 184) are shown in bold type. ***Identity = 26.0 %. Similarity = 48.5 %. Number of gaps = 7.***

An alignment between OrfU and the C-terminal domain of the *Saccharomyces cerevisiae* PF2K enzyme (PF2K-Y) is presented in Figure 4.2.7 and shows conservation of the same important functional residues with a slightly better general similarity.

S. cerevisiae fructose-2,6-bisphosphatase x OrfU

```
                .         .         .         .         .
PF2K 224 PKYIWLSRHGESIYNVEKKIGGDS__SLSERGFQYAKKLEQLVKESAGEI 271
 -Y       . :|| ||||.  |::  .:|..  .|..||:: |..|. |:.: . ::
OrfU   1 MMRLWLIRHGETQANIDGLYSGHAPTPLTARGIEQAQNLHTLLHGVSFDL 50
                .         .         .         .         .
     272 NLTVWTSTLKRTQQTANY.LPYKKLQWKALDELDAGVCDG___MTYEEIE 317
            |:.|.|.|.|:||.. |. :.|. . :.||:. . ::   . . ::
      51 ___VLCSELERAQHTARLVLSDRQLPVQIIPELNEMFFGDWEMRHHRDLM 97
                .         .         .         .         .
     318 KEYPEDFKARDNDKYEYRYRGGESYRDVVIRLEPVIM___ELERQENELI 364
         .| :|::.|: ||. .    .||:::...  |:|..|    |::: :| |:
      98 QEDAENYSAWCNDWQHAIPTNGEGFQAFSQRVERFIARLSEFQHYQNILV 147
                .         .         .         .         .
     365 ITHQAVLRCIYAYFMNVPQE________ESPWMSIPLH____TLIKLEPR 402
         :.||:||. :.| ::.:|.|        ::.| .|.::    ||  |:.|
     148 VSHQGVLSLLIARLIGMPAEAMWHFRVDQGCWSAIDINQKFATLRVLNSR 197
                .         .         .         .         .
     403 AYGTKVTKIKANIPAVSTYKEKGTSQVGELSQSSTKLHQLLNDSPFQDKF 452
         |.|.. .
     198 AIGVENA_____________________________________________ 204
```

Figure 4.2.7 *Alignment between the second half of PF2K-Y and OrfU.* Presentation is as in Figure 4.2.6. ***Identity = 29.0 %. Similarity = 51.9 %. Number of gaps =7.***

It was very interesting that all matches of OrfU with PGAMs, were between approximately the first seventy amino acids, including the phosphohistidine motif, and that all matches with PF2Ks identified an approximately 50 amino acid stretch centred around the important distant histidine residue.

One problem with these comparisons was the presence of some large gaps in the alignments due to OrfU being approximately 50 amino acids shorter than these enzymes. When OrfU was aligned with the uncharacterised OrfX mentioned above this was not a problem as they were similar sizes. Also, a recently sequenced PGAM from *Schizosaccharomyces pombe* (PGAM-P) was found to be smaller than all the other PGAMs and was of a similar size to OrfU and OrfX. When alignments were made with PGAM-P, numbers of identical and similar amino acids were comparable to previous alignments but gaps were

much smaller. Table 4.2.1 shows the results of alignments between all possible pairs of OrfU, OrfX, PF2K-Y, the rat liver PF2K which was found to be the most similar to OrfX (PF2K-R), PGAM-E, and PGAM-P.

Table 4.2.1 Sequence alignment data.

	PGAM-P	PGAM-E	PF2K-Y	PF2K-R	OrfX	OrfU
PGAM-P		49.3	24.6	26.9	24.1	25.1
PGAM-E	69.6 [1]		30.0	27.9	23.7	26.0
PF2K-Y	44.5 [6]	52.5 [9]		52.9	23.4	29.0
PF2K-R	52.2 [6]	50.5 [8]	68.8 [2]		28.9	26.2
OrfX	47.6 [7]	45.5 [7]	46.8 [7]	51.7 [9]		25.4
OrfU	46.7 [6]	48.5 [7]	51.9 [7]	54.5 [6]	56.2 [1]	

% Identity

% Similarity [Number of Gaps]

The alignments described in Table 4.2.1 prompted a few tentative conclusions. The position and sequence of the phosphohistidine motif and the accessory histidine residue were conserved in all the alignments and this alone seemed to confirm that the relationship between these enzymes and OrfU (and OrfX) was a significant one. Other residues known to be situated near or in the active site of the *S. cerevisiae* PGAM (Nairn *et al.*, 1994, Fothergill-Gilmore and Watson, 1989) were also conserved consistently.

The two known PGAM enzymes, despite one being eukaryotic and the other prokaryotic, were much closer to each other than to either OrfU or OrfX. Also, the two PF2K enzymes were very highly conserved with each other. This suggested that, although OrfU and OrfX were related to these two classes of enzymes, they were almost certainly functionally different.

There is an established relationship between PGAMs and PF2Ks based not only on sequence comparisons, but also from crystallographic and biochemical studies (Bazan *et al.*, 1989, Fothergill-Gilmore and Watson, 1989), so it was significant that OrfU was as similar to PGAMs and PF2Ks as they were to each other. It is not unreasonable to conclude that OrfU is probably a member of a

different class of enzymes related to both phosphoglycerate mutases and fructose-2,6-bisphosphatases. OrfX appeared to be slightly more similar to OrfU than to any of the other sequences (the alignment with PF2K-R containing nine gaps) but a similar conclusion to the one above could be made for it. Using the same criteria however, it would have to be a member of yet another family of enzymes.

It may be significant that OrfU was more similar to the PGAM-E than any of the eukaryotic PGAMs. This may be expected if OrfU is a protein which has evolved from PGAM-E to something similar.

The PGAM-P protein is unique amongst PGAMs because it acts as a monomer (Nairn *et al.* 1994). This is thought to be directly associated to its small size in the following way; in an alignment between PGAM-Y and PGAM-P, the *S. pombe* protein lacks a large region which has been shown to be involved in multimerisation of the *S. cerevisiae* protein. The PF2K enzymes lack the same region and act as monomers (Bazan *et al.*, 1989). Likewise OrfU and OrfX lack this region and the inference could be made that, if their relationship to PGAMs and PF2Ks is valid, OrfU and OrfX probably both act as monomers as well.

Motifs. A command from the GCG package called "Motifs" was used to look for established protein sequence motifs in the predicted sequences of both OrfUU and OrfU. The only result from this was the identification of the phosphohistidine motif in OrfU as discussed above.

4.2.3 Analysis of sequence data for the entire fifteen minute region

The completion of the sequence of the fifteen minute region allowed it to be analysed as a whole.

Database comparisons. Aside from *orfUU* and *orfU* there were four genes of unknown function in the fifteen minute region. These were all known to produce protein products; see Table 4.1.1. The sequences for *ybeB*, *ybeA*, *rlpB* and *rlpA* and their products were regularly put through the same database comparison programs as described for *orfUU* and *orfU*. None of the four genes harboured any similarity to any

known proteins except the previously documented lipoprotein signal sequences at the N-terminus of both *rlpA* and *rlpB* (Takahase *et al.*, 1987).

Transcriptional terminators. Two important question about the fifteen minute region were whether the *mrd*-operon was in fact an operon and, if so, where did it start. The proposed relation of *orfU* to a family of glycolytic enzymes appeared to further support the idea that the fifteen minute region consisted of two totally unrelated groups of genes: the *mrd*-operon (*ybeB*, *ybeA*, *pbpA*, *rodA*, *rlpA* and perhaps *dacA*) and another group consisting of *leuS*, *rlpB*, *holA*, *orfUU* and *orfU*. The latter group appeared to have no obvious link with each other however, the close proximity of their open reading frames and the lack of reasonable promoter consensus sequences upstream of *rlpB*, *holA*, *orfUU* or *orfU* indicated that they could constitute another operon, transcribed from a promoter upstream of *leuS*. This was tentatively named the *leuS*-operon.

The position of transcriptional terminators would support or refute the existence of two possible operons, that is, an operon would not be expected to contain a terminator, whereas the gap between two operons would be likely to. Sequence analysis can allow the identification of potential factor-independent terminators using a combination of the primary sequence and predicted secondary structure of the RNA products involved. The "terminator" command of the GCG package judges stretches of sequence firstly on the basis of their ability to form a stem-loop structure and secondly on the surrounding primary sequence, compared to that of known transcriptional terminators. Potential terminator sequences are those which score above threshold values for each of these two aspects. The whole region was assessed for possible transcriptional terminators with the following results.

[i] A good terminator sequence was found after the *dacA* gene. This consisted of a 10 base perfect inverted repeat separated by four bases, which would form a stem-loop. It also had an adenine and thymine rich stretch 3' to the stem-loop structure. The GCG program gave this sequence high scores in both aspects.

[ii] All other sequences identified by the GCG program as possible terminators scored considerably lower than the *dacA* terminator. This was thought to limit their significance although terminators can be weak. One of these potential terminators was present between *orfU* and *ybeB*, exactly where it would be predicted if there were two separate operons. It consisted of a four base inverted repeat separated by five bases. With two bases in one side of the stem pushed out there was another four base repeat, giving potential for a stem of 8 bases. The next 20 bases 3' to the potential stem-loop contained 18 adenines or thymines. This sequence starts at bp 1485 in Figure 4.2.2 and its potential structure is shown in Figure 4.2.8 compared to the probable terminator after *dacA*.

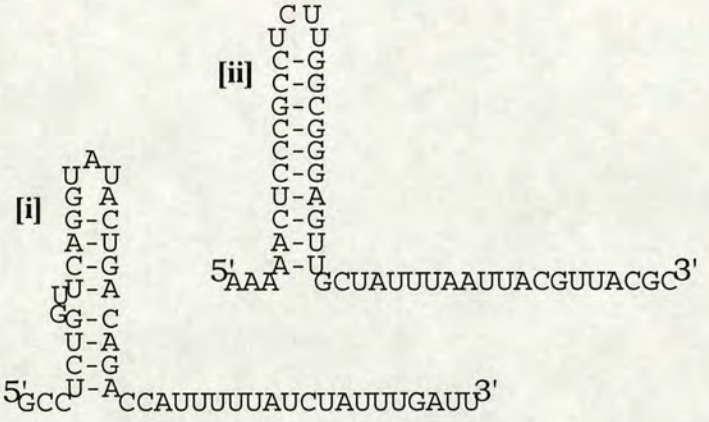


Figure 4.2.8 *Secondary structure of terminators.* 'U's are included instead of 'T's as these are potential RNA structures. Base pairs in the stem-loop structures are shown by horizontal lines. [i] Shows the potential terminator between the *leuS*-operon and the *mrd*-operon. [ii] Shows the probable terminator after *dacA*.

Although this seemed like a reasonable terminator it only contained four G-C base pairs. Also, other sequences were picked out by the computer which were in unfavourable positions for the two operon idea (in *leuS*, *holA*, *rlpA* and *dacA*). From the computer prediction, some of these were better candidates for factor-independent terminators than the one illustrated above, however

the computer did not pick out the extra four base pairs introduced by the pushing out of two bases in one arm of the stem. This feature was picked out by eye and it may therefore be an unlikely one.

[iii] In a separate search two sequences were found upstream of *ybeB* which were one base away from consensus DnaA boxes (Schaefer and Messer, 1989). They were within four bases of one another in opposite orientations (bp 1374–1382 and 1395–1387 in Figure 4.2.2). It is known that DnaA boxes can act as transcriptional terminators *in vivo* (Schaeffer and Messer, 1989, Wende *et al.*, 1991).

[iv] The gap between *orfU* and *ybeB* of 367 bp contains no significant open reading frames and many stop codons. It was thought that this stretch of untranslated DNA was a potential site for Rho-dependent transcription termination. At present, it is impossible to predict the presence of such terminators because the sequence elements involved are only very loosely defined (Platt, 1994).

Encouraged particularly by the results in [ii] and [iii] a search for an active trancriptional terminator between *orfU* and *ybeB* was instigated. The preliminary results of this search are described in section 4.2.4.

Codon usage. Codon usage in *E. coli* (and many other organisms) has been shown to differ significantly and consistently between highly and poorly expressed genes (for example Grosjean and Fiers, 1982, Sharp and Li, 1987). A correlation has therefore been drawn between codon usage and efficiency of translation. Two factors are proposed to be involved in this (Grosjean and Fiers, 1982).

[i] Codons which are recognised by transfer RNAs (tRNAs) of low abundance or those which are very weakly interacting, are rare in highly expressed genes. In order for fast and efficient translation, codons recognised by abundant and/or moderately interacting tRNAs are preferred. In poorly expressed genes these codons are far more frequent, although still rare.

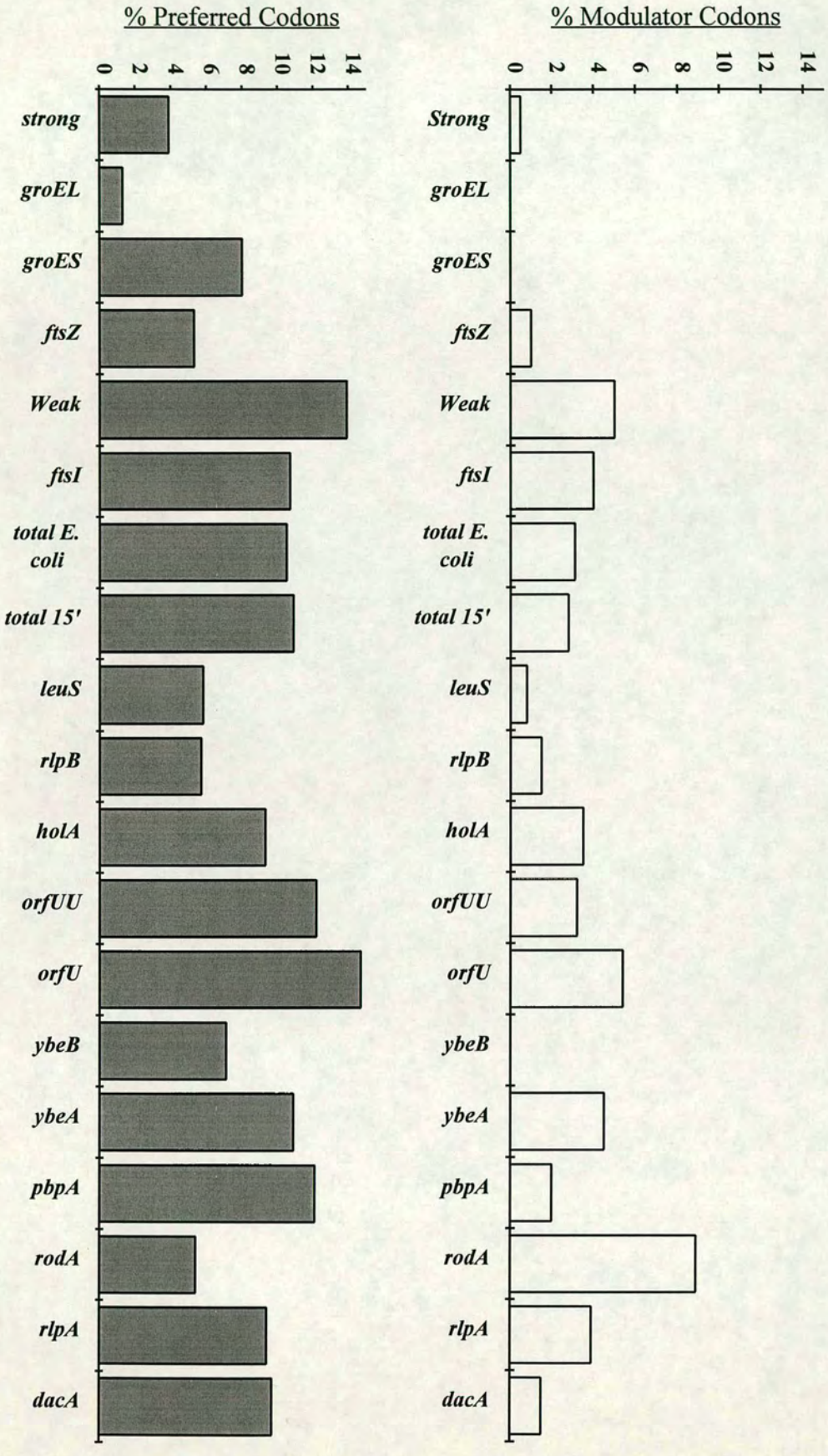
[ii] In the genes for highly expressed proteins, there is a tendency to preferentially choose codons which will give moderate codon/anticodon interactions. For example, if the first two nucleotides in a codon are Gs or Cs, which have strong interactions with their complement then, if degeneracy and third base 'wobble' allows, the final base will be an A or a T, which have weak interactions. It is thought that the presence of very strongly or very weakly interacting codon/anticodon pairs is deleterious to the translation process. There are therefore pairs of degenerate codons of which one (the moderately interacting) is preferred in highly expressed genes and the other (the weakly or strongly interacting) predominates in poorly expressed genes.

It has therefore become common practice to infer something about the level of expression of new genes by looking at their codon usage (for example Dong *et al.*, 1993). Using the table describing modulator codons and other codons preferentially used in poorly expressed genes from Grosjean and Fiers (1982), the codon usage for the whole fifteen minute region was analysed. The results are presented in Figure 4.2.9 and discussed at length in the accompanying legend.

Briefly, none of the products of the uncharacterised genes or open reading frames were expected to be highly expressed proteins, with *rlpB* probably being the most abundant. OrfU showed the characteristics of a particularly poorly expressed protein.

Figure 4.2.9 *Codon usage in the fifteen minute region compared with highly and poorly expressed proteins.* The figure shows the usage of two different types of codon which are specifically avoided in highly expressed proteins and conversely present in above average amounts in poorly expressed proteins. These are described in the preceding text. The upper graph shows the proportion of modulator codons as a percentage of the total number of codons in each protein or group of proteins. These were described in Grosjean and Fiers (1982) and are CUA, AUA, CGA, CGG, AGA, AGG, GGA and GGG. The lower graph shows the frequency of a group of codons which are preferentially used in poorly expressed genes due to providing either a very high or very low codon/anticodon energy of interaction (Grosjean and Fiers, 1982; see text). This is also expressed as a percentage of the total number of codons. It appears that the upper graph is a better general guide to the potential level of expression of a gene; see below. Calculations have also been made for some controls: the group of highly expressed genes used in Grosjean and Fiers (1982) along with three individual examples *groEL*, *groES* and *ftsZ*; the group of poorly expressed genes (Grosjean and Fiers, 1982) along with an individual example *ftsI*; the average for all codons in Genbank63 from *E. coli* (excluding duplicates, pseudogenes, mutant and synthetic genes, JM Cherry, personal communication) and an average for the fifteen minute region.

Penicillin-Binding assays consistently show higher levels of PBP5 than PBP2 (Spratt, 1977, Spratt, 1980, Stoker et al., 1983a, DH Edwards, 1994) in fact PBP5 is thought to be present in approximately 1800 molecules per cell as opposed to 20 for PBP2 (Spratt, 1977). These differences appear to be reflected in the codon usage (see also Asoh *et al.*, 1986), although one might expect PBP2 (PbpA) to have more rare codons as Spratt (1977) showed it to be less abundant than PBP3 (FtsI). The product of the *holA* gene is a subunit of the DNA polymerase III holoenzyme which is only present in 10-20 copies per cell (Dong *et al.*, 1993). It is thought to be a poorly expressed gene and this is reflected to a certain extent in the codon usage. Both *rlpB* and *rlpA* were thought to be rare lipoproteins (hence the name) by their discoverers (Takase *et al.*, 1987). The codon usage would suggest that *rlpA* is less well expressed than *rlpB* but that the latter is possibly quite well expressed. It must be remembered that these two genes were called rare with respect to other lipoproteins which are amongst the most abundant proteins in the cell. From this study *leuS* would appear to be a highly expressed gene which is not surprising for the tRNA synthetase for the most abundant amino acid in *E. coli* proteins. The presence of a high proportion of modulator codons in *rodA* suggests it is a very poorly expressed gene however the lower graph does not support this. From expression studies of *rodA*, Stoker *et al.* (1983) concluded that it was probably a poorly expressed gene in vivo, suggesting that the upper graph is more reliable. The *ybeB* gene is very small and so its codon usage is likely to be misleading. The *ybeA* gene is unlikely to be a highly expressed gene. The region of DNA containing *holA*, *orfUU* and *orfU* was present in the 'maxi-cells' protein-labelling experiments of Takase *et al.* (1987) and was thought not to produce any proteins. This suggests that these three genes are poorly expressed and as with *holA*, the codon usage in *orfUU* and *orfU* is consistent with that prediction. One would predict that *orfU* has the poorest expression of the three.


% Preferred Codons
0
2
4
6
8
10
12
14
strong
groEL
groES
ftsZ
Weak
ftsI
total E. coli
total 15'
leuS
rlpB
holA
orfUU
orfU
ybeB
ybeA
pbpA
rodA
rlpA
dacA
% Modulator Codons
0
2
4
6
8
10
12
14
Strong
groEL
groES
ftsZ
Weak
ftsI
total E. coli
total 15'
leuS
rlpB
holA
orfUU
orfU
ybeB
ybeA
pbpA
rodA
rlpA
dacA

4.2.4 Detection of a transcriptional termination activity in the gap between the leuS-operon and the mrd-operon

As mentioned in the previous section, experiments were instigated to test whether a terminator existed between the two prospective operons. The presence of one in this position would support the two operon idea. Given further time, this approach would also have been applied to the regions in which terminators would not be expected, in order to support the following data.

PCR product. The region to be studied for the presence or absence of transcriptional termination activity was amplified by the PCR reaction. PCR was carried out as described in Chapter 2 (*2.2.8*) using pADD30 as a template. (In a control reaction, where chromosomal *E. coli* DNA was used as a template, the same size PCR product was obtained.) PCR primers were designed with single mismatches which introduced *Hind*III sites at either end of the final product, allowing it to be cloned easily. Figure 4.2.10 shows the sequence of the PCR product and restriction sites used in clonings described in the next section.

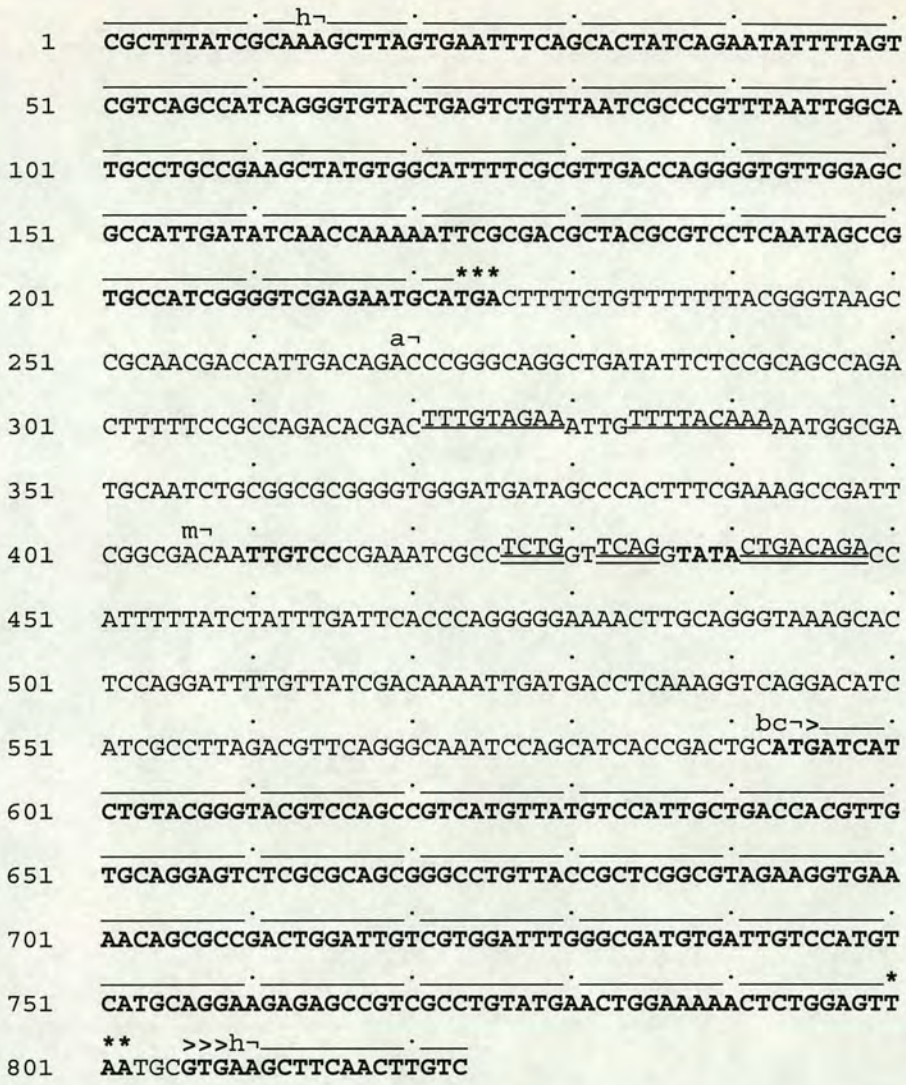


Figure 4.2.10 *PCR product containing the gap between the leuS-operon and the mrd-operon.* The sequence of the 823 bp region amplified by PCR is shown. The non-coding strand is given, so RNA sequence would be as written (substituting Us for Ts). Nucleotides are numbered from the start of the product (equivalent to base 1098 from Figure 4.2.2) and dots above the sequence are at every ten bases. Open reading frames are shown in bold type with a horizontal line above. Start codons are indicated by > and stop codons by *. Relevant restriction sites are marked with ¬ and the letter: a - *AvaI* (at bp 270), bc - *Bcl*I (at bp 594), h - *Hin*dIII (at bp 14 and 810) or m - *Mun*I (at bp 407). Two possible terminator sequences are in superscript. The computer predicted one (*4.2.3[ii]*) is doubly underlined and the two DnaA boxes (*4.2.3[iii]*) are singly underlined. Each primer contained a mismatch such that bp 14 and 814 were changed from G to A and G to T respectively, to form the two *Hin*dIII sites. The proposed promoter sequences described in the following text are also shown in bold type.

Plasmid constructs. In order to assay for transcriptional termination activity various fragments of the PCR product were cloned into pJW30. This vector was designed specifically for detecting termination activity (Wright *et al.*, 1992). Essentially, pJW30 contains the *E. coli galE* promoter region (p*galE*) upstream of the otherwise promoterless *galK* reporter-gene. Between promoter and reporter-gene there are restriction sites for *Hin*dIII and *Bam*HI and translational stop codons in all three reading frames. The idea is that DNA containing a transcriptional terminator, will reduce or stop expression of the *galK* gene if it is cloned between the promoter and the reporter-gene. The expression of *galK* can be assayed directly (described in Newman *et al.*, 1982), however for this preliminary study the colour of transformant colonies of a *gal*$^-$ strain, on M^{c}Conkey-galactose agar plates, was used as a rough guide. Cells expressing *galK* on these plates gave red colonies whereas those with no *galK* expression were white.

Different fragments of the PCR product mentioned above were cloned into pJW30 to form the plasmids pADD59, pADD60, pADD61, pADD62, pADD63 and pADD64. The construction of these plasmids is described briefly in Chapter 2 (*2.1.3*) and is shown in more detail in Figure 4.2.11 along with the structure of the relevant region of pJW30. A complicating feature of these experiments was the potential presence of a promoter upstream of *ybeB* proposed to drive expression of that gene, *ybeA*, *pbpA*, *rodA* and *rlpA*. This promoter was predicted by Asoh *et al.* (1986) from the nucleotide sequence and its activity has been detected by the same group (Matsuzawa *et al.*, 1989). Its predicted position is bp 410-415 (-35 box) and 437-440 (-10 box) in Figure 4.2.10. The distance between these two sequences (22 bp) is normally considered to be outside the limits (16–18) for a promoter, however pADD63 was constructed in order to confirm or refute its presence.

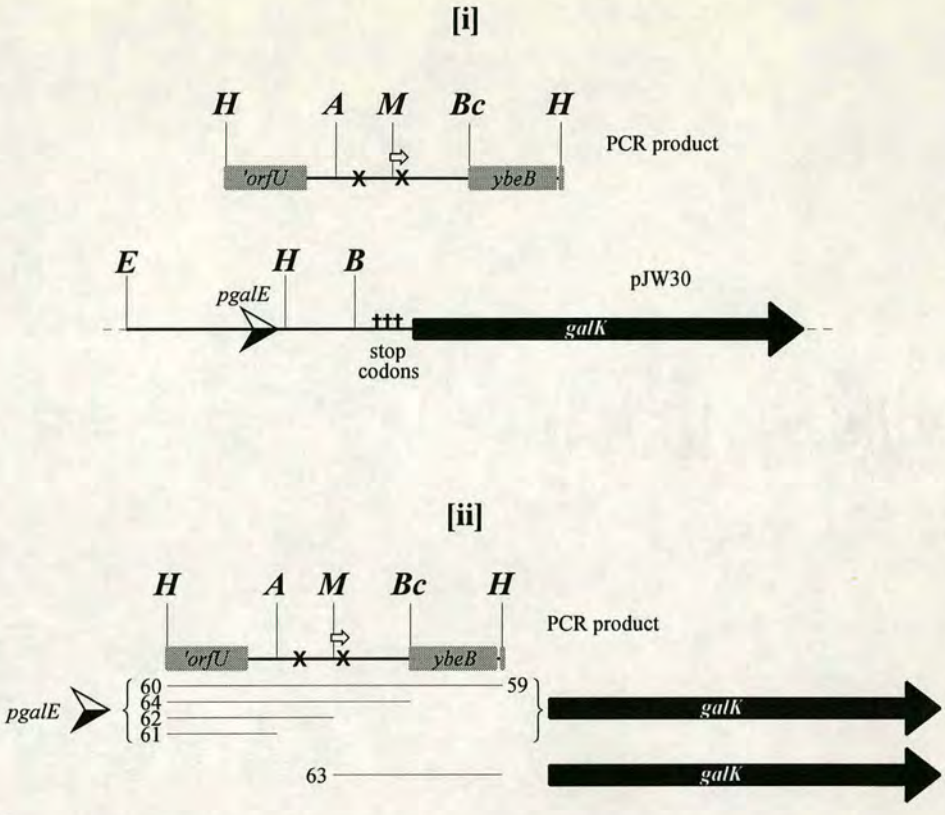


Figure 4.2.11 *Construction of plasmids for transcriptional termination assays.* In [i] the 823 bp PCR product from Figure 4.2.10 is shown with the appropriate region of pJW30. In the PCR product, two '**X**'s mark the positions of the potential terminators, the potential *mrd*-operon promoter (see text) is marked with ⇨ and the end of *orfU* (*'orfU*), *ybeB* and the first 18 bp of *ybeA* are shown as grey boxes. In pJW30 p*galE* is represented by ➢, the translational stops in all three forward reading frames are indicated by '✝'s and the *galK* reporter-gene is shown as a black arrow. Relevant restriction sites are labelled as follows: *A* - *Ava*I, *B* - *Bam*HI, *Bc* - *Bcl*I, *E* - *Eco*RI, *H* - *Hin*dIII, and *M* - *Mun*I.

In [ii] the inserts in each of the constructs used in the terminator assay experiment are shown as lines under the PCR product. pADD59 and pADD60 were the result of simple cloning of the PCR product into the *Hin*dIII site of pJW30 in either orientation. pADD64 similarly was the result of cloning the *Hin*dIII/*Bcl*I fragment of the PCR product into a *Hin*dIII/*Bam*HI restriction of pJW30. The other plasmids resulted from further restriction and religation of pADD60, as described in Chapter 2 (*2.1.3*), leaving the indicated fragment upstream of *galK*. The diagram shows that for pADD59, 60, 61, 62 and 64 p*galE* is present but in pADD63 it has been removed by using the upstream *Eco*RI site. This was done in order to leave the potential *mrd*-operon promoter upstream of *galK* by itself. [A sister vector to pJW30 (pKO1, Wright *et al.*, 1992) does not have the *galE* promoter and gives no expression of *galK*. Therefore this approach is valid for the detection of promoter activity.]

Results. The six plasmids containing fragments of the PCR product, pJW30 and a promoterless version of pJW30 (pKO1) were transformed into strain X.[6] It has been recorded that constructs with very high *galK* activity can actually produce colonies which are paler than expected. This is due to the accumulation of promoter down mutations to reduce the *galK* expression (RS Hayward, personal communication). Constructs initially producing excessive amounts of GalK are easily identified because transformants produce sectored red/white colonies when they are first streaked onto McConkey-galactose plates. They also grow poorly, overexpression of *galK* apparently being detrimental to the cells. In this work it was noticed that transformants of strain X with pADD60 had this sectored colony phenotype and showed particularly poor growth. It was important therefore to use fresh transformants for each repetition of the experiment to reduce the chance of accumulation of promoter down mutations (which would have a similar effect to the presence of a terminator, on colony colour). By recording the colour of individual colonies in all repetitions it was observed that no mutations of this type effected the results presented below, that is, the photographs in Figure 4.2.12 reflect the initial *galK* expression of all clones.

Figure 4.2.12 shows transformants of strain X with pADD59–64, pJW30 and pKO1 and strain X by itself, after overnight incubation on McConkey galactose agar. Figure 5.2.13 displays part of Figure 5.2.11 along with the simplified results from Figure 5.2.12. The implications of the results are discussed in the legend.

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[6] The strain used for these assays is not included in the strain table in Chapter 2 (*2.1.1*) for the following reason. The strain was obtained from K McKenny via RS Hayward via S Dewar as C600 *gal*$^-$. It was subsequently found to be both *thr*$^+$ and *his*$^-$, neither of which corresponded to C600 (see 2.1.1). Ths strain was definately *gal*$^-$, as judged by the its white colonys on McConkey-galactose plates, and so it was used for these experiments, however its provenance remains unknown. It is called strain X in the text.

Figure 4.2.12 *Terminator assay experiments.* All photographs were taken with the same exposure and aperture settings. The darkness of colonies is a direct result of the amount of red pigment they contained. The key indicates the plasmid present in the strain X colonies on each plate. All plates also contained Amp except for that with strain X by itself. Transformant colonies were resuspended in LB, the OD_{600} measured and then dilutions made such that the plating of 200 µl of suspension would give separate colonies.

The controls showed that strain X was white, pKO1 gave white colonies and pJW30 gave dark red colonies. The 'red' or 'white' nature conferred by each plasmid is obvious from the photographs except that pADD59 consistently gave small, faintly pink colonies. It can be seen that although the pADD60 transformant was diluted similarly to the others, it gave fewer and smaller colonies which were very dark. This was thought to be due to very high expression of *galK* from this construct. Other red colonies appeared to be similar in size to those containing pJW30 and were deemed to have a similar magnitude of *galK* expression to that plasmid. The implications of these results are discussed in detail in the text.

The one red colony on the pADD64 plate is difficult to explain. It is possible that this indicates that the white colonies in fact have very high *galK* expression and this red colony is a promoter down mutation. This would go directly against the original conclusions from this experiment, however, the white colonies on the same plate show no signs of poor growth or sectoring and so this explanation is probably wrong. It may be that the pADD64 plasmid itself is unstable for some unrelated reason and that the insert is lost at a low frequency.


59
60
62
63
61
pKO1
64
pJW30
strainX

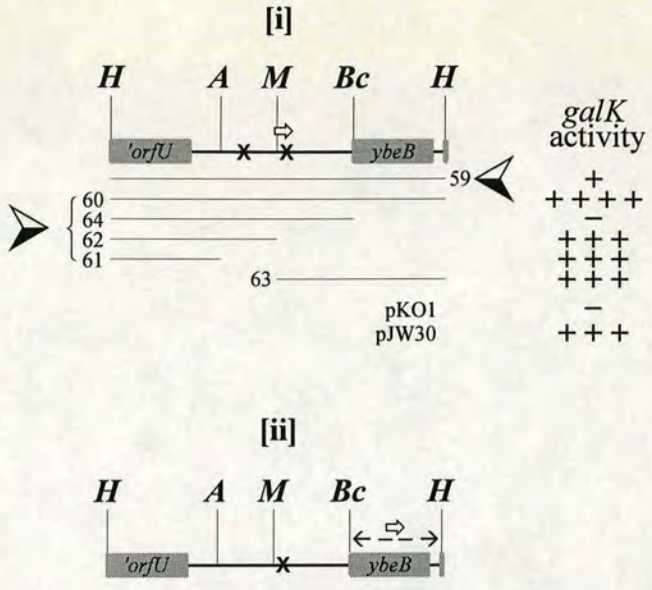


Figure 5.2.13 *Results of terminator and promoter assays in the gap between the leuS-operon and the mrd-operon.* In [i], the results of the assays are summarised next to the original diagram of the constructs with pJW30 and pKO1 as controls. The colonies were split into four classes in Figure 5.2.12: those with more activity than pJW30 (pADD60), similar activity to pJW30 (pADD61, 62 and 63), less activity than pJW30 (pADD59) and similar activity to pKO1 (pADD64). These are represented by plus and minus signs accordingly.

Simple but important conclusions can be made from these results. pADD63 shows that there is a promoter between *Mun*I and the distal *Hin*dIII. pADD64 shows that there is a terminator between the proximal *Hin*dIII and *Bcl*I. The promoter in pADD63 is not present in pADD64 and so must be between *Bcl*I and the distal *Hin*dIII (or cut by *Bcl*I). pADD61 and 62 detect no termination between the proximal *Hin*dIII and *Mun*I and therefore the terminator must be between *Mun*I and *Bcl*I (or cut by *Mun*I). Low *galK* expression in pADD59 is consistent with two promoters acting against each other upstream of the reporter gene. Activity from pADD60 confirms the presence of a promoter downstream of the terminator, however it is difficult to see why that activity would be higher than in pADD63.

In [ii], the conclusions from these results are summarised. There is a terminator between *Mun*I and *Bcl*I (or perhaps straddling the *Mun*I site). This may correspond to the computer predicted terminator described above (*4.2.3*). There is a promoter between *Bcl*I and the proximal *Hin*dIII (or perhaps straddling the *Bcl*I site). This does not correspond to the promoter sequences predicted by Asoh *et al.* (1986) but could be that detected by (Matsuzawa *et al.*, 1989). The promoter appears to be similar in strength to p*galE*.

4.3 Discussion

The two new open reading frames identified in this work appear most likely to be in a single transcriptional unit with *leuS*, *rlpB* and *holA*, - the *leuS*-operon. Codon usage suggests that the expression of these proteins gradually decreases along the operon. This could be achieved by differential translation of the same mRNA. One of the new open reading frames shows no similarity to any previously published protein or DNA sequences, however the other, *orfU*, is clearly related to a family of glycolytic enzymes. Preliminary data from terminator and promoter assay experiments supports the idea of two operons separated by an untranslated gap containing a terminator. It also suggests that a strong promoter, which probably drives expression of *pbpA*, is situated in the *ybeB* gene. It is unclear, therefore whether *ybeB* is expressed *in vivo*.

There is vast opportunity for further characterisation of the transcriptional and translational organisation of this region. Directly related to the experiments described here, is the possibility of other assays, using smaller fragments of the PCR product thought to contain the terminator and promoter separate from one another.

Chapter 5

Genetic and Molecular Analysis of the Genes of the Fifteen minute region

5.1 Introduction

The sequencing of an open reading frame is one of the many possible first steps in the discovery of a new gene. One could also start with a mutant strain or a purified protein, both of which would possibly be better indications of the biological significance of that gene. The sequencing approach however, is becoming more and more common due to the current ease with which large regions of DNA can be sequenced, and the establishment of sequencing projects for the genomes of many organisms, including *E. coli*. Much information can be gleaned from the analysis of sequence alone (see Chapter 4), however, when an open reading frame is identified, it is important to demonstrate that it has a biological function. Obvious approaches would be to: [i] show the production of a protein from the open reading frame and then perhaps purify and investigate the activity of the protein, [ii] look at the effects of overexpressing the gene in wild-type *E. coli* cells and [iii] attempt to isolate mutations in that gene and study their phenotypes.

Complete sequence analysis of the fifteen minute region led to the discovery of two new open reading frames, at least one of which was thought to be a true gene, due to the similarity of its predicted product to a well characterised family of enzymes (*Chapter 4*). The entire region therefore potentially contained six genes or open reading frames of unknown function: *rlpB*, *orfUU*, *orfU*, *ybeB*, *ybeA* and *rlpA*. The likelihood that these were split into two unrelated operons, one containing genes involved in a number of general biosynthetic processes and the other involved specifically in cell-wall synthesis and morphology, became clearer as the work progressed; see Chapter 4. However, a comprehensive attempt was made to initiate characterisation of all of these genes, in some of the ways mentioned above. Particular emphasis was placed on the two *rlp* genes, which were proposed by Takase *et al.* (1987) to be involved in cell-shape maintenance, and the newly sequenced *orfUU* and *orfU* open reading frames. The extent of this characterisation process for each of the genes is described in this chapter.

5.2 Protein Products of the Uncharacterised Genes

5.2.1 Introduction

The protein products of the *rlpB*, *rlpA*, *ybeB* and *ybeA* genes have all been identified by using 'maxi-cells' and minicells protein-labelling procedures (Spratt *et al.*, 1980, Stoker *et al.*, 1983a, Takase *et al.*, 1987). It was of interest to this work that in many of these published experiments, constructs which also contained *orfUU* and *orfU* were used and appeared not to produce any proteins. It should be noted that the *holA* gene, which has turned out to be an important gene for a component of the replication machinery of *E. coli*, was also present in these studies without its product being detected. It was decided that both *orfUU* and *orfU* should be cloned separately to allow more specific protein-labelling experiments to be carried out. Another reason for attempting to construct plasmids containing these genes (and the other four uncharacterised genes) was to observe the effects of their overexpression in wild-type *E. coli*, for potential clues to their function. A good example of this approach is that the tenfold overexpression of the *dacA* gene, causes wild-type *E. coli* cells to grow as spheres, eventually killing them (Markiewicz *et al.*, 1982). In this case the enzymatic function of the gene product (PBP5) was known beforehand, but from the extreme morphological defect, it would have been logical to suggest a function in cell wall synthesis.

5.2.2 Identification and overproduction of proteins

Genes of the fifteen minute region were cloned from λ15D7, a phage from the Kohara collection (Kohara *et al.*, 1987) containing DNA from the middle of the *dacA* gene to well upstream of the *leuS* gene. A plasmid, pADD30, which contained a large fragment from λ15D7 was also used for many subclonings. All plasmid descriptions and a restriction map of the region (*Figure 2.1.1*) are given in Chapter 2 (*2.1.3*).

RlpB. A *Mun*I fragment containing the *rlpB* gene was cloned, in either orientation, into the *Eco*RI site of pUC19, to form pADD1 and pADD2. Subsequent publications and this work revealed that these plasmids also contained *holA*, *orfUU* and *orfU*. The pUC19 cloning vector has

an extremely high copy number and its polylinker lies downstream of the inducible *lac* promoter (p*lac*). This allows high levels of transcription to be induced through a cloned piece of DNA, by the addition of IPTG. The high copy number itself is a convenient way of overexpressing a gene *in vivo* and induction can be used to visualise its protein product; see Chapter 2 for methods (*2.5.1*).

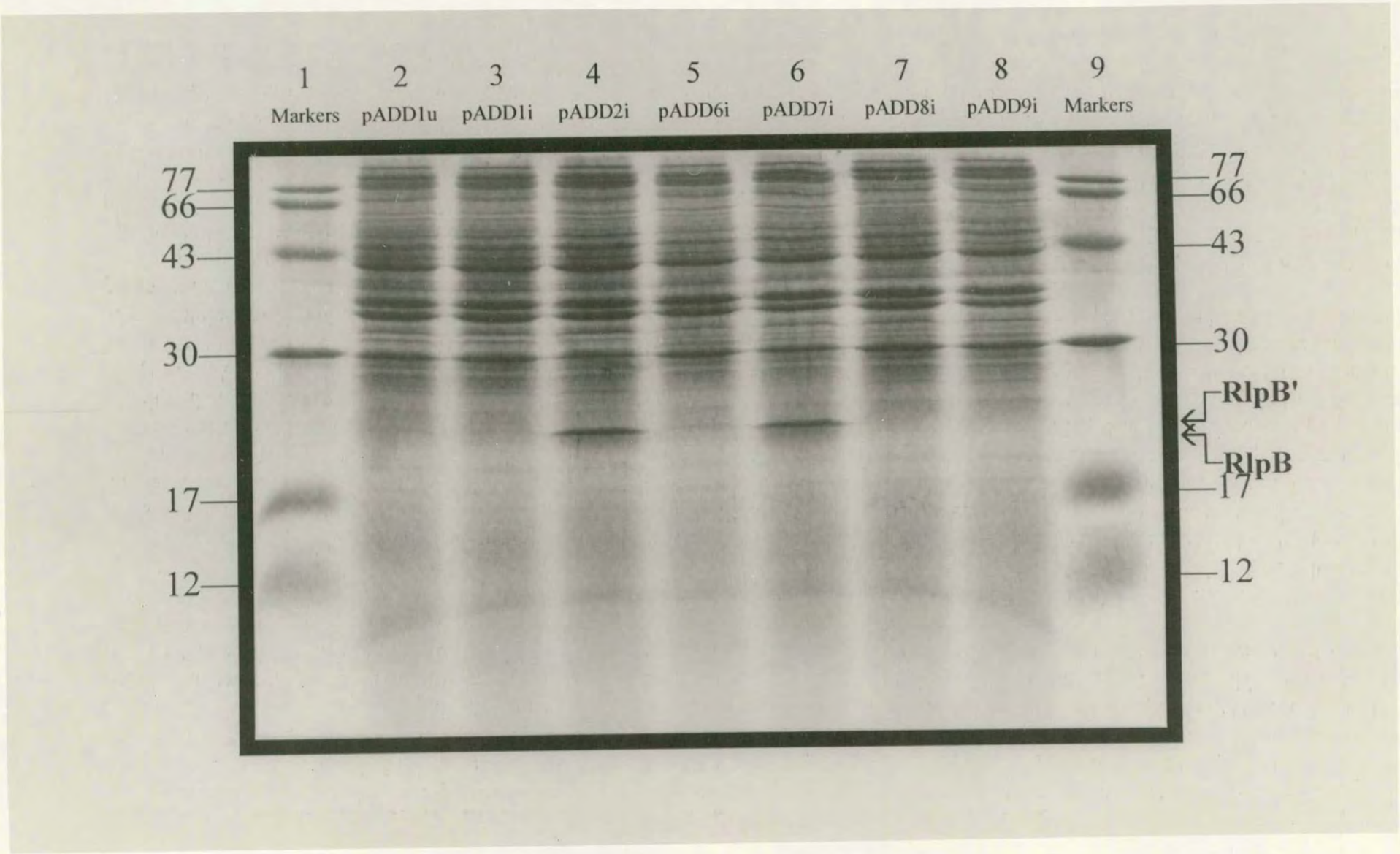
The effect of overexpressing *rlpB* was tested by comparing cultures of DH5αF'I^Q containing pADD2 (which has the gene cloned in the correct orientation to be expressed from p*lac*) with the same strain containing pUC19. It was noted that cells grew slightly less well when they contained pADD2, however there were no differences in the appearance of the cells when observed microscopically. This deleterious effect was found not to be due to overexpression of *rlpB* however, because similar plasmids (described below) where *rlpB* was insertionally inactivated, had the same effect. Experiments described in section 5.3.2 suggested that the same effect was observed with clones containing only *holA*. A plasmid containing *rlpB* by itself in moderately high copy number (pADD46) had no noticeable effect on host cells.

The omega (Ω) fragment of pHP45Ω (*2.1.3*) was cloned into the *Bcl*I site in the middle of the *rlpB* gene in pADD1 and 2 to form pADD8 and 9 respectively. As a control, the Ω fragment was also cloned into the *Bgl*II site in the unsequenced region of pADD1 and 2 to form pADD6 and 7 respectively; sequencing subsequently showed that this site was in *orfUU* (*Chapter 4*). The proteins produced from pADD1, pADD2 and their insertion derivatives were investigated by the methods described in Chapter 2 (*2.5.1*). Figure 5.2.1 shows the resulting Coomassie Blue-stained protein gel, the main result being the very efficient overproduction of a protein with a molecular weight of about 22 kD. This protein corresponded to RlpB which has a predicted molecular weight of 19.445 kD. The band was present in plasmids which had *rlpB* intact and disappeared when *rlpB* was interrupted by the Ω fragment, thus confirming that RlpB had been overproduced and that insertions into the *Bcl*I site stopped its production completely. More detailed implications of this protein gel are discussed in the legend accompanying Figure 5.2.1.

Figure 5.2.1 *Overproduction of RlpB and disruption of the cloned gene.* Protein samples were prepared as described in Chapter 2, denatured at 100°C for 5 min in Sample Buffer and loaded onto a denaturing 15% polyacrylamide gel. Molecular weight markers were as described in Chapter 2 (*2.5.1*) and are labelled at either side. All samples were made from DH5αF'I^Q cultures containing the plasmid indicated above each lane. "u" indicates an uninduced sample (-IPTG) and "i" indicates an induced sample (+IPTG for 3 h). The uninduced lane was run as a control; however, preliminary gels showed that, with DH5αF'I^Q containing pUC plasmids grown in LB, IPTG made no difference to the profile of bands observed. This may not have been because induction made no difference, but because induction was taking place 'naturally' in the LB.

The very clearly overproduced protein running at about 22 kD in lanes 4 and 6 is the product of the *rlpB* gene. This is confirmed by the disappearance of the band in lanes 7 and 8 where the *rlpB* gene is disrupted by the Ω fragment. The plasmid in lane 6 has the Ω fragment inserted elsewhere (in *orfUU*) which does not affect the band. Close inspection of the gel reveals that the same band is present in lanes 2, 3 and 5 indicating that *rlpB* is being expressed in opposition to p*lac*, albeit at much lower levels. (It is worth repeating that even this faint band is not present in lanes 7 and 8, thus confirming that it is RlpB). The lack of this same band in lanes 7 and 8 may seem confusing because *rlpB* was present on the chromosome of the host cells. Two possible explanations for this are, [i] Chromosomal expression of *rlpB* is not sufficient to produce a band in this experiment or, [ii] being a lipoprotein, RlpB is synthesised as a preprotein which is then cleaved to produce the mature protein. The two different versions of RlpB were distinguished on protein gels by Takase *et al.*, (1987). A band below the heavily overproduced band of lanes 4 and 6 can be seen, which is strongest in these lanes but also seems to be present in all other lanes. If this is the mature form of RlpB then the most prominent band is the preprotein (RlpB'). The accumulation of preprotein would probably be due to saturation of the processing enzyme. Therefore there may be RlpB produced from chromosomal genes on the gel, but it is all in the mature form.

The presence of extra RlpB on the gel even when the gene is cloned in the opposite orientation to p*lac* could indicate either [i] that *rlpB* actually has its own promoter between the *Mun*I site used to clone it and the start of the gene, or [ii] that there is another promoter in pUC19 which is in the opposite orientation to p*lac*. The latter explanation fits better with the idea of a *leuS*-operon however promoter assay experiments would be needed to answer this question. No bands corresponding to HolA, OrfUU or OrfU are visible on this gel. In particular there is no evidence of the disappearance of OrfUU when its gene is disrupted by the Ω fragment. This is further support for the suggestion that expression of both *orfUU* and *orfU* may be very poor (as for *holA*) compared to that of *rlpB* for example; see Chapter 4 (*Figure 4.2.9*). These results also imply that the limiting factor for poor expression would be translation, because in this experiment very high levels of transcription through the genes has been forced.


1
Markers
2
pADD1u
3
pADD1i
4
pADD2i
5
pADD6i
6
pADD7i
7
pADD8i
8
pADD9i
9
Markers
77
66
43
30
17
12
77
66
43
30
RlpB'
RlpB
17
12

RlpA. The *rlpA* gene was cloned on a *Kpn*I/*Hin*dIII fragment[7] into pUC18 such that its expression could be directed from p*lac* (pADD11) and a control plasmid with the fragment in the opposite orientation was made by repeating the cloning into pUC19 (pADD10). These plasmids also contained the *rodA* gene; this was confirmed by showing that they could complement *rodA* mutants.

Similar experiments to those described for *rlpB* were carried out for *rlpA*, using pADD10, 11, 13 and 14; pADD14 was pADD11 with an internal fragment of *rlpA* removed; pADD13 had the same fragment replaced by the Ω fragment. Samples were denatured gently, by incubation at 37°C in Sample Buffer, because of previous observations that RodA does not enter the gel matrix if samples are boiled (Stoker *et al.*, 1983b). No bands corresponding to the predicted size of RlpA (35.614 kD or its previously observed size on two different gel systems 54 kD or 43 kD, Takase *et al.*, 1987) or RodA, were observed in Coomassie Blue-stained protein gels. It was known that at least the *rodA* gene was being expressed from these clones (due to their complementation of *rodA* mutants) so a more specific method of protein visualisation was used. Fragments containing *rlpA* and *rodA*, and some with disruptions of *rlpA*, were cloned into pT7-5, a vector from which inserted DNA can be transcribed by the phage T7 RNA polymerase. This system allows specific radioactive pulse-labelling of the cloned genes because transcription of all host genes can be stopped by the addition of rifampicin, to which the T7 RNA polymerase is resistant. The results of specific radioactive labelling experiments are shown in Figure 5.2.2 and discussed in detail in the accompanying legend. Both RodA and RlpA were detected, their sizes being consistent with previously published data (Stoker *et al.*, 1983b, Takase *et al.*, 1987), and it was shown that disruption of the *rlpA* gene caused its product to disappear. As with the overproduction of RlpB, both the mature RlpA protein and the unprocessed preprotein were visible.

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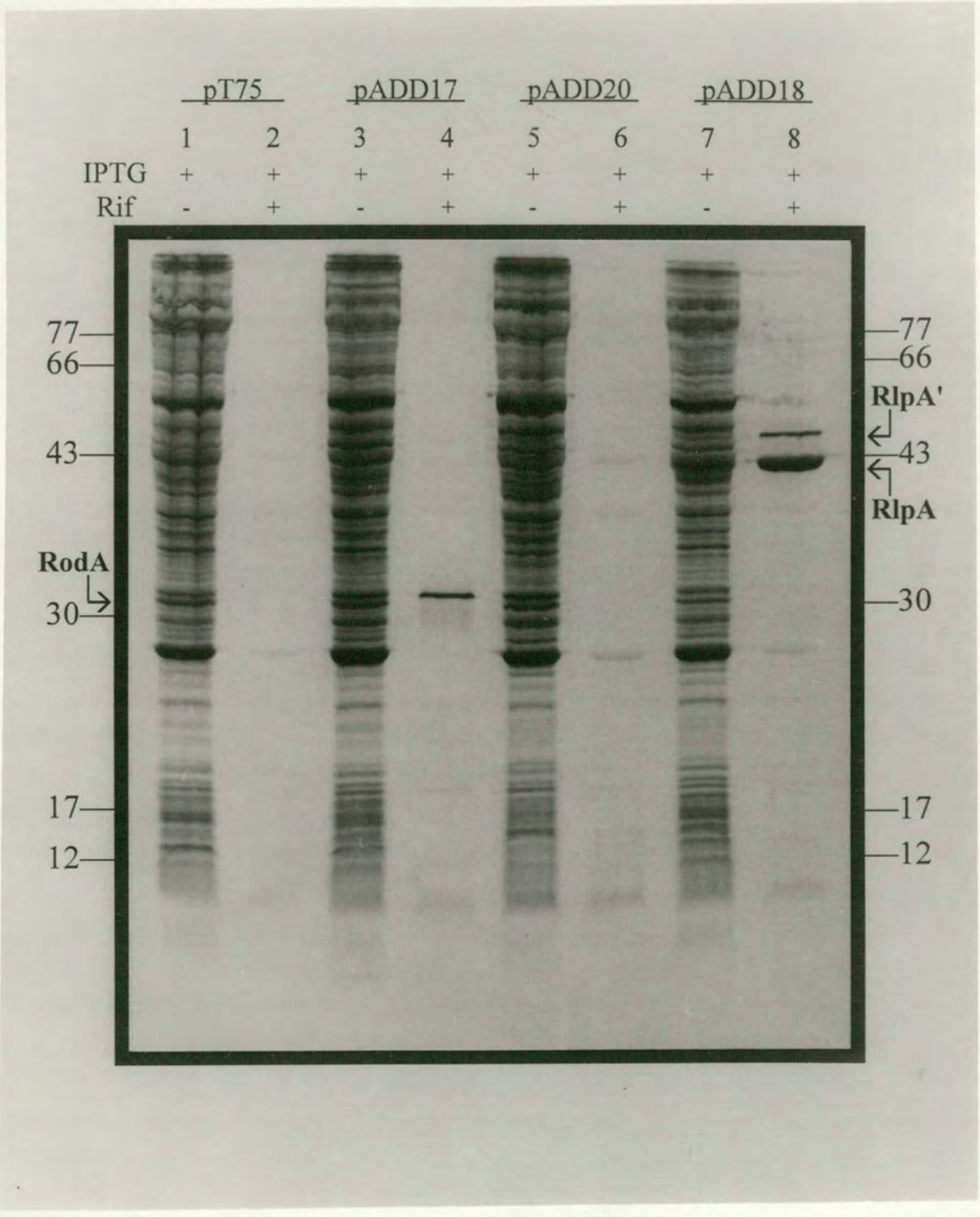
[7] The *Hin*dIII site came from the pACYC184 part of pADD5, from which it was sub-cloned.

Figure 5.2.2 *Specific radioactive labelling of RlpA and RodA and disruption of the cloned rlpA gene.* Protein samples were prepared as described in Chapter 2 (*2.5.1*), denatured at 37°C for 1 h in Sample Buffer and loaded onto a denaturing 10% polyacrylamide gel. Molecular weight markers were as described in Chapter 2 and their positions are marked at the side of the gel. Experimental samples were prepared from BL21(DE3) cultures containing the plasmid indicated above each lane. Addition of IPTG or rifampicin, as described in Chapter 2, is indicated above each lane.

Lane 8 shows the specific labelling of RlpA, running at about 42 kD, and its slightly larger unprocessed form (RlpA'), from pADD18 which contains *rlpA* only. Lane 6 shows the absence of both bands from pADD20, which is pADD18 with 0.77 kb removed from the middle of *rlpA*. In pADD17, the 0.77 kb *Bam*HI internal *rlpA* fragment has been replaced by the Ω fragment. This plasmid also contains *rodA* and consequently, lane 4 shows RodA at about 31 kD, but not RlpA.


pT75
pADD17
pADD20
pADD18
1 2 3 4 5 6 7 8
IPTG + + + + + + + +
Rif - + - + - + - +
77
66
43
RodA
30
17
12
77
66
RlpA'
43
RlpA
30
17
12

The clear difference in the ease with which the two Rlp proteins were detected (that is, RlpB>>RlpA) supported the prediction, based on codon usage, that *rlpB* is better expressed than *rlpA in vivo*; see Chapter 4.

No effect was observed on the morphology of DH5αF'I^Q cultures containing *rlpA* in high copy number, however when pADD11 (*rodA*, *rlpA*) and pADD13 (*rodA*, *rlpA*::Ω) were used to complement spherical mutants it was found that those containing pADD11 grew slower, perhaps indicating that excess RlpA has a deleterious effect on cell growth.

YbeB and YbeA. No attempt was made to overexpress *ybeB* and *ybeA* from p*lac*.[8] However, *ybeA* was cloned into the pT7-6 vector (pADD53) and its product was specifically radioactively labelled in a similar experiment to that described for *rlpA*. This is illustrated in Figure 5.2.3 and discussed in the accompanying legend.

No effect was observed on the morphology or growth of DH5αF'I^Q cultures containing these two genes on a moderately high copy number plasmid (pADD35), or on the BL21(DE3) cells where its overproduction was artificially induced from pADD53.

OrfUU and OrfU. Attempts to identify proteins from *orfUU* and *orfU* by expressing them from p*lac* were unsuccessful (see RlpB above). Therefore it was decided to use the T7 system, as described for *rlpA* and *ybeA*. (Unfortunately this has not yet been possible for *orfUU*, due to cloning difficulties.) The results for *orfU* are shown in Figure 5.2.4. A protein of approximately 25 kD was specifically labelled from the plasmid containing *orfU* (pADD46) and this band was absent in the controls. The size of the band corresponded well with the 23.44 kD predicted size of OrfU. Thus it was shown that the *orfU* open reading frame encoded a protein, further evidence for it being a gene.

---

[8] Most plasmids were made during complementation studies described in section 5.3 and a plasmid containing these two genes alone (pADD35) had already been made.

Figure 5.2.3 *Specific radioactive labelling of YbeA.* Protein samples were prepared as described in Chapter 2 (*2.5.1*), denatured at 37°C for 1 h in Sample Buffer and loaded onto a denaturing 10% polyacrylamide gel. Molecular weight markers were as described in Chapter 2 and their positions are marked at the side of the gel. Experimental samples were made from BL21(DE3) cultures containing the plasmid indicated above each lane. Addition of IPTG or rifampicin, as described in Chapter 2, is indicated above each lane.

Lane 8 shows YbeA specifically labelled at just over 17 kD. This is absent in the equivalent vector only control (lane 4). Other bands in lane 8 are due to reduced effectiveness of rifampicin in that sample. The same bands are feinter, but present, in controls (lanes 3, 4 and 7). *ybeB* was also present in pADD53, however a *Bcl*I site in the ATG codon of this gene was used during the cloning, thus removing the rbs. Hence, there is no 7 kD band corresponding to YbeB in lane 8. The T7 vectors are not as high in copy number as the pUC vectors, however it is worth noting that YbeA could not be distinguished on the Coomassie Blue-stained gel before autoradiography, supporting inferences from codon usage, that it is not a highly expressed gene.

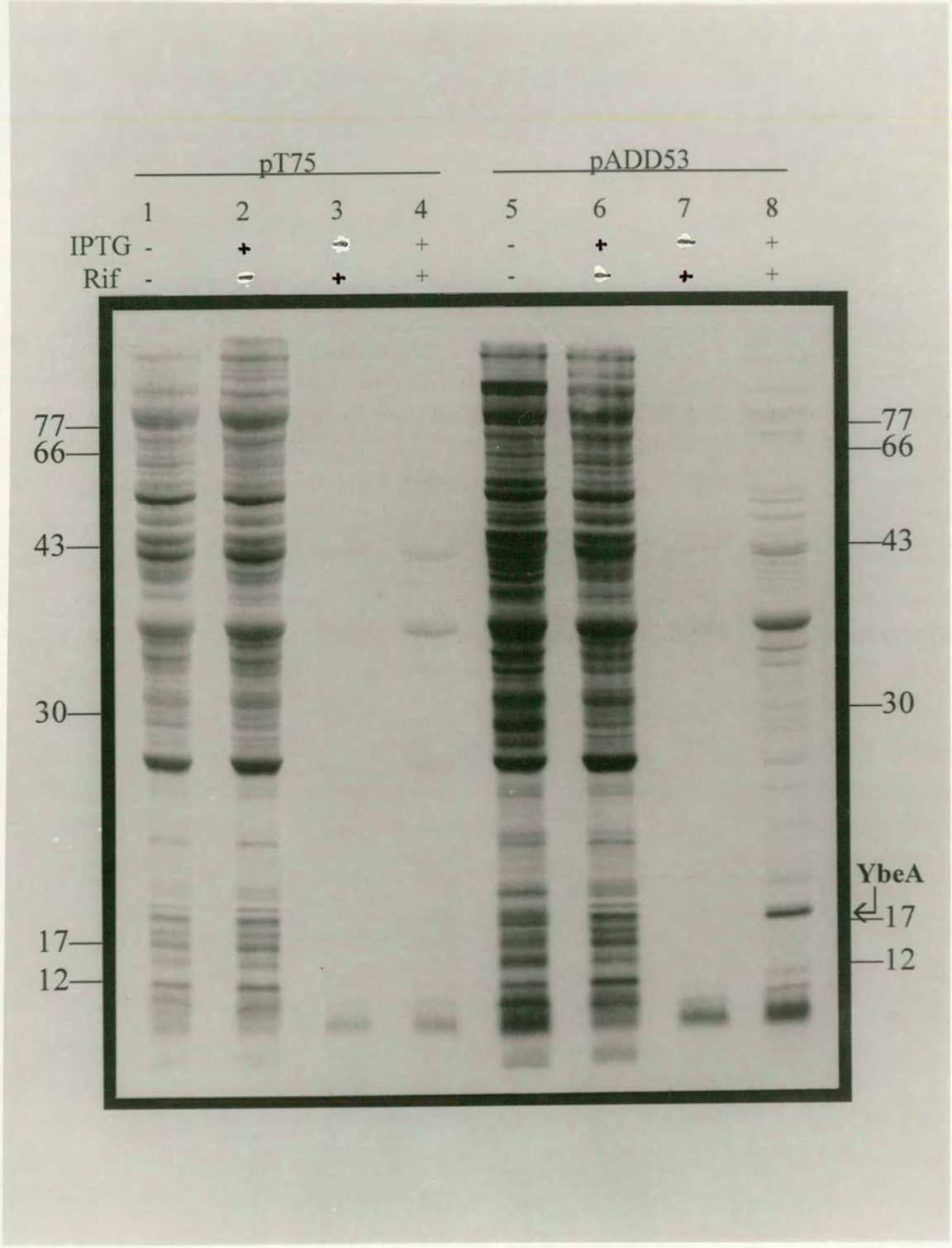
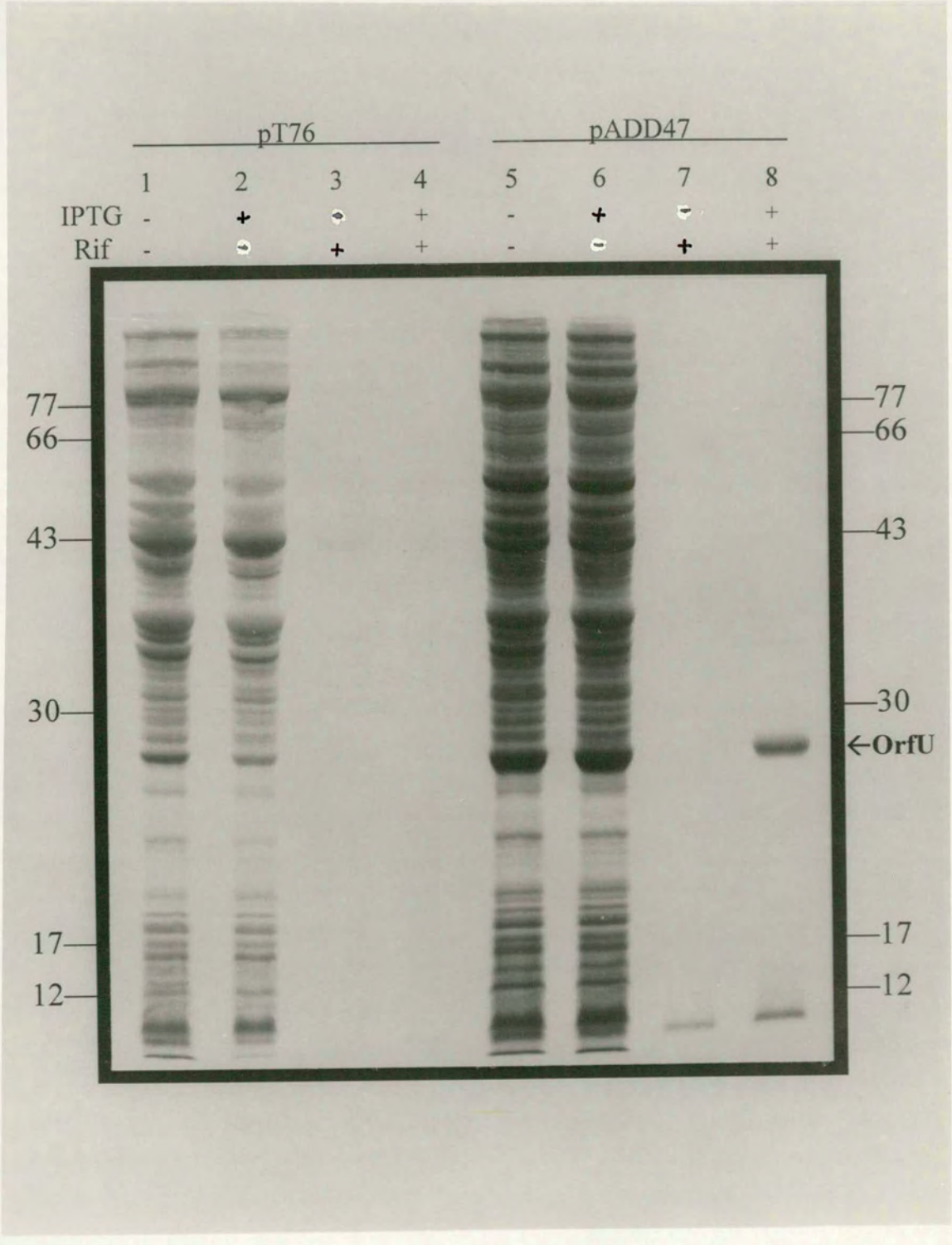

pT75
pADD53
1 2 3 4 5 6 7 8
IPTG - + - + - + - +
Rif - - + + - - + +
77
66
43
30
17
12
77
66
43
30
YbeA
17
12

Figure 5.2.4 *Identification of OrfU, the product of the orfU gene.* Protein samples were prepared as described in Chapter 2 (*2.5.1*), denatured at 37°C for 1 h in Sample Buffer and loaded onto a denaturing 10% polyacrylamide gel. Molecular weight markers were as described in Chapter 2 and their positions are marked at the side of the gel. Experimental samples were made from BL21(DE3) cultures containing the plasmid indicated above each lane. Addition of IPTG or rifampicin, as described in Chapter 2, is indicated above each lane.

Lane 8 shows the specific labelling of OrfU, the previously unidentified product of the *orfU* gene, from pADD47. This band is not present in any of the control plasmid samples (pT7-6, lanes 1-4) or the uninduced samples of pADD47 (lanes 5, 6). The size of the band was calculated by plotting the migration distance of marker proteins on semi-log paper and then interpolating. This was done for two separate gels giving the values 25.0 and 25.5 kD. This is in good agreement with the 23.4 kD predicted from the sequence.


pT76
pADD47
1 2 3 4 5 6 7 8
IPTG - + - + - + - +
Rif - - + + - - + +
77
66
43
30
17
12
77
66
43
30
←OrfU
17
12

The only basis for a formal name for *orfU*, was its similarity to PGAMs and PF2Ks. It was therefore tentatively named *phpB* (**ph**osphohistidine **p**rotein) with the suggestion that *orfX* might be named *phpA* as it could be described in similar terms and was discovered first.[9]

Cultures in which expression of *orfU* was induced, grew less well than the vector only control. This meant that overexpression of *orfU* was mildly deleterious to cells and could have contributed to the deleterious effect of pADD2 (see previous section on RlpB).

5.2.3 Discussion

Studies of the proteins expressed from uncharacterised genes and open reading frames in the fifteen minute region showed that [i] RlpB could be easily overproduced with no detectable effect on cells. Future work could utilise this fact to purify the protein for antibody production or biochemical studies. [ii] RlpA was more difficult to overproduce than RlpB and in extreme high copy number, its gene appeared mildly detrimental to cell growth. [iii] YbeA was only detectable when specifically radio-labelled indicating that, similar to *holA*, *orfU*, and *rlpA*, *ybeA* was probably a poorly translated gene. [iv] the open reading frame *orfU* encoded a protein, which is good evidence for it being a gene, and was detrimental to cell growth when overexpressed.

[9] For the sake of continuity, the rest of this thesis will continue to refer to *orfU*.

5.3 Random Mutagenesis of the Fifteen Minute Region

5.3.1 Introduction

A genetic approach to the characterisation of the genes of the fifteen minute region was now taken. A technique of localised random mutagenesis was chosen in an attempt to produce mutants of genes in the region. The technique, developed by Hong and Ames (1971), involved transduction with mutagenised phage P1 lysates. The lysates were made on strains SHA76 and CAG12149 (*2.1.1*) which had Tn*10* transposons either side of the fifteen minute region (in SHA76 the transposon was closest to *leuS* and in CAG12149 it was closest to *dacA*). Transduction with one of these lysates and selection for tetracycline resistance, resulted in the recipient cells receiving mutated chromosomal DNA specifically surrounding the transposon. Methods for hydroxylamine mutagenesis (*2.4.5*) and P1 transduction (*2.4.6*) are described in Chapter 2.

The recipient strain used for transductions with mutated lysates, was OV2. It was chosen because of the presence of a *supF*^{ts} mutation which would allow the detection of amber mutants as well as mis-sense temperature sensitive (*ts*) mutations. Transductions were carried out at 30°C and plated at the same temperature on NBT plates supplemented with thymine (NBTthy). Screening was done by patching transductant colonies on to NBTthy plates at 30 and 42°C. Potential *ts* mutants were streaked to single colonies and then re-tested for *ts* on NBthy plates, with and without Tet. It was important to ascertain that the mutant strains were *ts* without Tet present, to eliminate the possibility of isolating a *ts* or amber mutation in the Tet^{R} gene of the transposon.

The presence of double mutants was considered unlikely by Gibbs *et al.* (1992) when using the same technique with lysates mutagenised for 24 h. Therefore lysates were mutagenised for 24 h in these experiments, except where stated. Some new mutations were 'purified' by P1 transduction back into OV2 or into W3110, however this seemed unnecessary due to the above statement. Also the localised nature of the original mutagenesis meant that cotransduction frequencies between two mutations in a double mutant would be very high.

Characterisation of new mutants was by complementation with a variety of plasmids containing DNA from the fifteen minute region. The plasmids were specific such that mutants in all genes could be distinguished, except for *ybeB* and *ybeA* which were not separated on any of the clones. The inserts in these plasmids are illustrated in Figure 5.3.1.

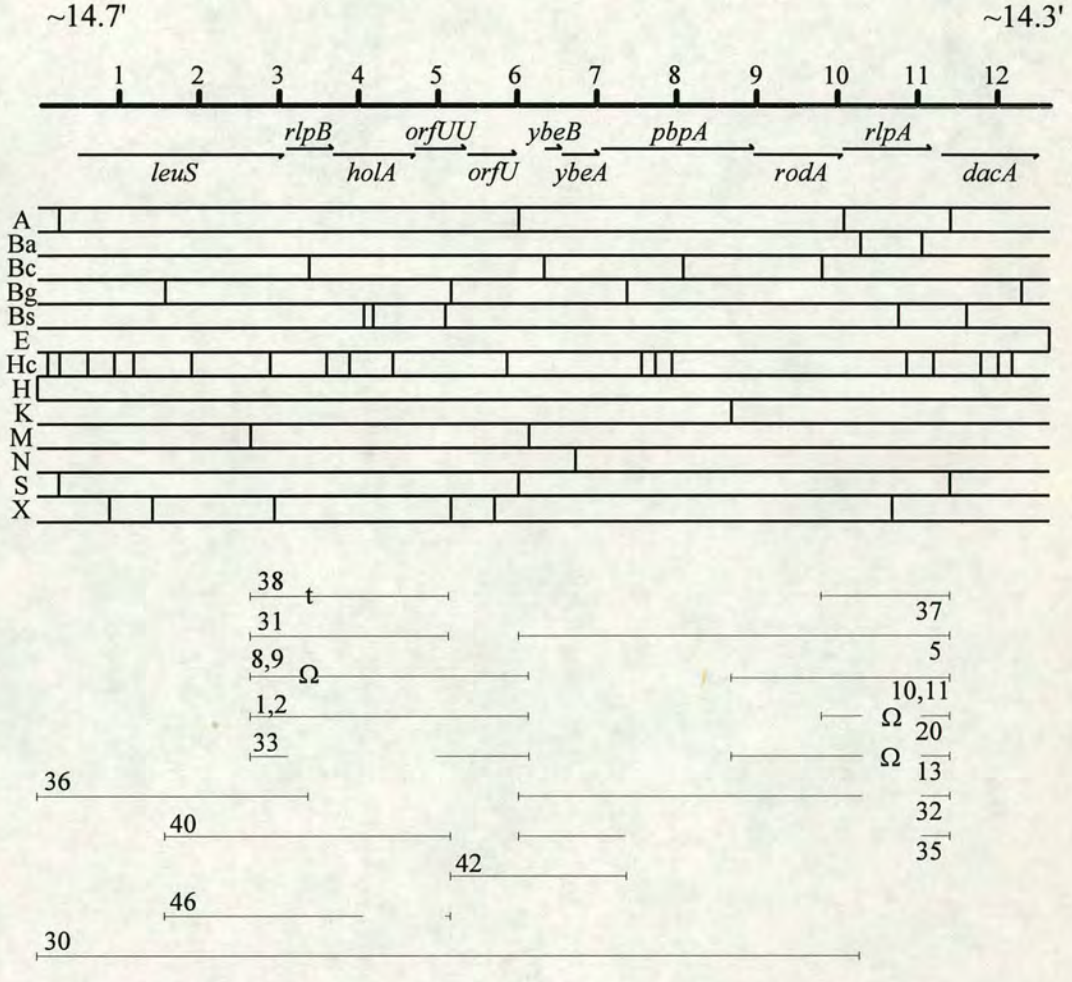


Figure 5.3.1 *Plasmids used for complementation of fifteen minute mutations.* The insert DNA in the plasmids used in the complementation studies of new mutants is illustrated below a reproduction of Figure 2.1.1. Horizontal lines bordered by two vertical lines represent the DNA in one plasmid. The number next to each line represents the 'pADD' number of the plasmid, as described in Chapter 2 (*2.1.3*). A break in the line indicates a deletion. 'Ω' indicates the presence of the Ω fragment. 't' indicates the presence of the Tet^R gene from pBR322. The cloning strategy for each plasmid is described in Chapter 2 (*2.1.3*).

The combined length of the uncharacterised genes in the fifteen minute region (~4.7 kb out of 12.7 kb) accounted for 37% of the sequenced area and it was hoped that a similar proportion of the mutants isolated in the region would be in these genes.

5.3.2 Results

Approximately 5000 transductants were screened (data combined for both lysates), and 30 colonies were identified, and then confirmed, as having *ts* behaviour. Ten of these were found to be *ts* only on plates containing Tet and so were thought to be Tet^{ts}. Fourteen of the remaining mutants were tested for whether they were due to amber mutations or not. This was done by making lysogens (*2.2.2*) with a lambda phage carrying the *supF* gene (λ*supF*). Only three of the fourteen mutants were shown to be suppressed by λ*supF*, suggesting that most had mis-sense *ts* mutations rather than ambers. The low number of amber mutants may have been for the following reasons: [i] Hydroxylamine is a very specific mutagen. It reacts with cytosine and causes the specific replacement of a G by an A, thus causing GC to AT transitions (Freese, 1971). This can potentially give 33 different amino acid substitutions. Conversely, only the TGG (Tryptophan) and CAG (Glutamine) codons can be mutated to give TAG amber codons. [ii] The positive results with the λ*supF* could also be reduced slightly because *supF* puts in tyrosine residues at TAG codons, so it is possible that some mutations which were from glutamine to amber would not be suppressed at high temperature by the addition of a tyrosine. [iii] The efficiency of the *ts* suppressor in OV2 ($supF^{ts}$) is only 10% even at the permissive temperature (Smith *et al.*, 1970) and it replaces ambers with glutamine or tyrosine in equal proportion. Some amber mutants therefore may not have survived to produce colonies on the transduction plates, due to receiving the correct amino acid at a frequency of only 5%.

All mutant strains are described in Chapter 2 (*2.1.1*) except for the Tet^{ts} strains which were not analysed further. Those mutations which were transduced in using lysates from SHA76 were given the prefix 'FI' (the transposon was nearest **fi**fteen minutes) and those transduced in with lysates from CAG12149 were given the prefix 'FO' (**fo**urteen minutes). Mutants FI99, FI139, FI145, FI164, FI606, FI625

and FI691 were all transduced with a lysate which had been mutated for 48 h.

The results of the complementation of these mutants are described below. The plasmids pADD30 and pADD5 were used to check first that the mutants were complemented by the fifteen minute region DNA available on plasmids. The relevant gene was then identified by selective use of the plasmids with smaller inserts. The results are shown both diagrammatically and in table form, the former to give a visual idea of the complementation strategy and the latter to show which strains were checked with which plasmids.

Mutants in characterised genes. Most of the mutants produced by this screen were found to be in previously characterised genes.

[i] Strains FI145, 342, 709, 866 and 1629 were mutated in *leuS*. Their complementation results are described in Figure 5.3.2 and Table 5.3.1.

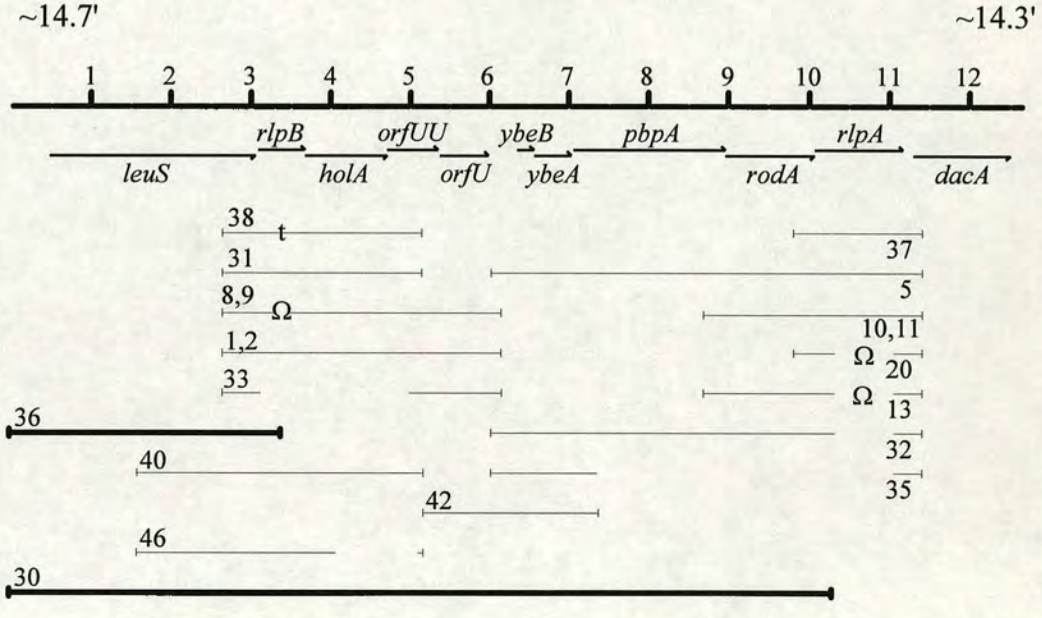


Figure 5.3.2 *Complementation results for leuS mutants.* In an adaptation of Figure 5.3.1, bold lines indicate plasmids which complemented strains FI145, 342, 709, 866 and 1629. Plasmids in feint lines were either not used or did not complement; see Table 5.3.1.

Table 5.3.1 Complementation results for *leuS* mutants.

		Mutant strain:				
		FI145	FI342	FI709	FI866	FI1629
Plasmid pADD:	1			–		
	2	–				
	5	–				–
	11		–			
	30	+	+	+	+	+
	31		–			
	33		–			
	35		–			
	36	+	+	+	+	
	38		–			
	40			–	–	–
	42			–	–	–
		Complementation: +, yes; –, no.				

These mutants characteristically showed very clean *ts* behaviour. At the non permissive temperature, on agar plates, any cells left at the inoculum were very slightly longer than normal and phase bright. They appeared not to have lysed. These mutants were not characterised further, except that lysogens of FI145, 709 and 886 with λ*supF* were shown to remain *ts*, suggesting that those mutations were not ambers.

[ii] Strains FI99, 164, 606, 625, 1216, 1625 and FO499, 544, 702 and 1022 were mutated in *pbpA* (there is a minor possibility that FO499 and 544 are *ybeA* or *ybeB* mutants but their reluctance to be transformed by pADD35 c.f. other non-complementing plasmids suggests that this is unlikely). Their complementation results are described in Figure 5.3.3 and Table 5.3.2.

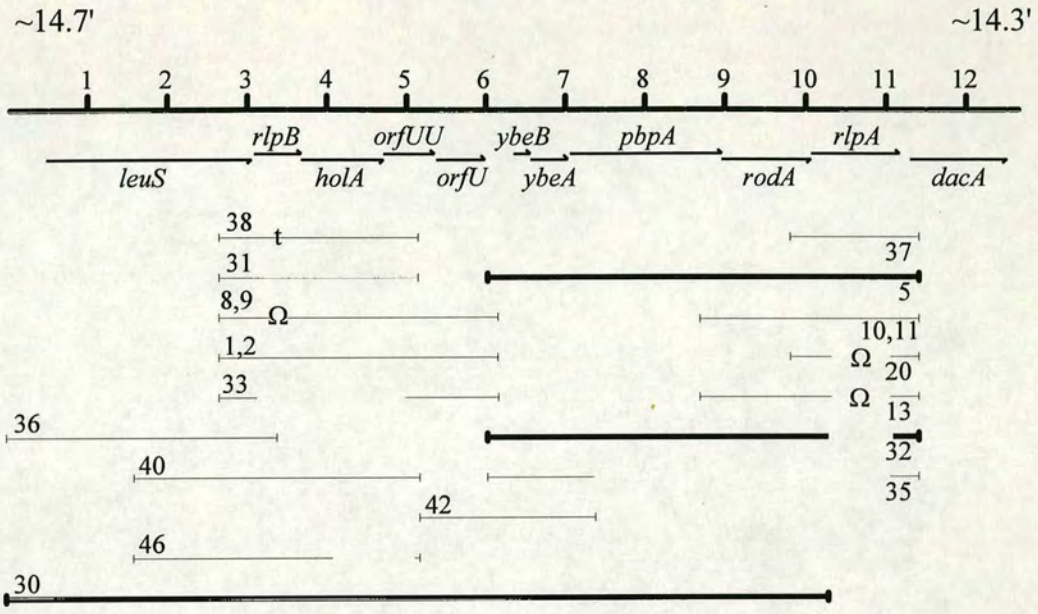


Figure 5.3.3 *Complementation results for pbpA mutants.* In an adaptation of Figure 5.3.1, bold lines indicate plasmids which were found to complement strains FI99, 164, 606, 625, 1216, 1625 and FO499, 544, 702 and 1022. Plasmids in feint lines were either not used or did not complement; see Table 5.3.2.

Table 5.3.2 Complementation results for *pbpA* mutants.

		Mutant strain:						
		FI99	FI164	FI606	FI625	FI1216 FI1625	FO499 FO544	FO702 FO1022
Plasmid pADD:	5	+	+	+	+	+		
	11	–	–	–	–			
	10						–	–
	13	–				–		
	20	–						
	30					+		
	32	+	+	+	+		+	+
	35	–	–	–	–		nt	–
	37	–		–				
	38	–						
	42	–		–	–	–		

Complementation: +, yes; –, no;
nt, would not transform.

These mutants had three different phenotypes. Group A: FI606 (an amber mutant) and FI625 were spherical at 30°C but lysed at 42°C. Group B: FI99, 1216, 1625 and FO544, 702 and 1022 were fat rods at 30°C which became spherical and lysed at 42°C. Group C: FI164 and FO499 were rod shaped at 30°C and spherical at 42°C but were viable at the higher temperature. These last two mutants were noticed as a result of slightly poorer growth and distinctly paler patches at 42°C. The latter feature appears to be common for spherical *E. coli* (this work) and spherical *S. typhimurium* mutants (Costa and Antón, 1993).

It has been reported that loss-of-function mutations in *pbpA* are lethal, unless certain specific suppressor mutations arise (Ogura *et al.*, 1989, Vinella *et al.*, 1992a). This remains disputed (KJ Begg, unpublished), spherical mutants apparently being stable on poor media without suppressors. The isolation of Group A and Group C type mutants which appeared to be stably spherical at 30°C and 42°C respectively, on NB media, could also dispute this view. It is possible that some of these were not completely null mutations, however FI606 was complemented by λ*supF* and so was an amber mutant; in most circumstances this would give a null phenotype for that gene. The idea that some of the other mutants were perhaps leaky, could be checked by penicillin-binding protein labelling assays. Another proposed property of *pbpA* mutants is a block to division (Vinella *et al.*, 1993). The presence of some filamentous but very bloated cells in some of the *pbpA* mutants of type B, at the permissive temperature, could support this. The presence of the three types of *pbpA* mutant described above, could indicate mutations which have varying effects on the activity of PBP2 at the permissive temperature. One could suggest that the activity of the mutant enzymes in Group A were lower than those in Group B which were in turn lower than those in Group C. This is a simplistic view, however biochemical characterisation, to compare the level of PBP2 activity (or the amount of PBP2 present) in each group combined with sequencing the mutations responsible for the different phenotypes, could be interesting.

[iii] Strains FI139 and FI205 were mutated in *holA*. Their complementation results are described in Figure 5.3.4 and Table 5.3.3.

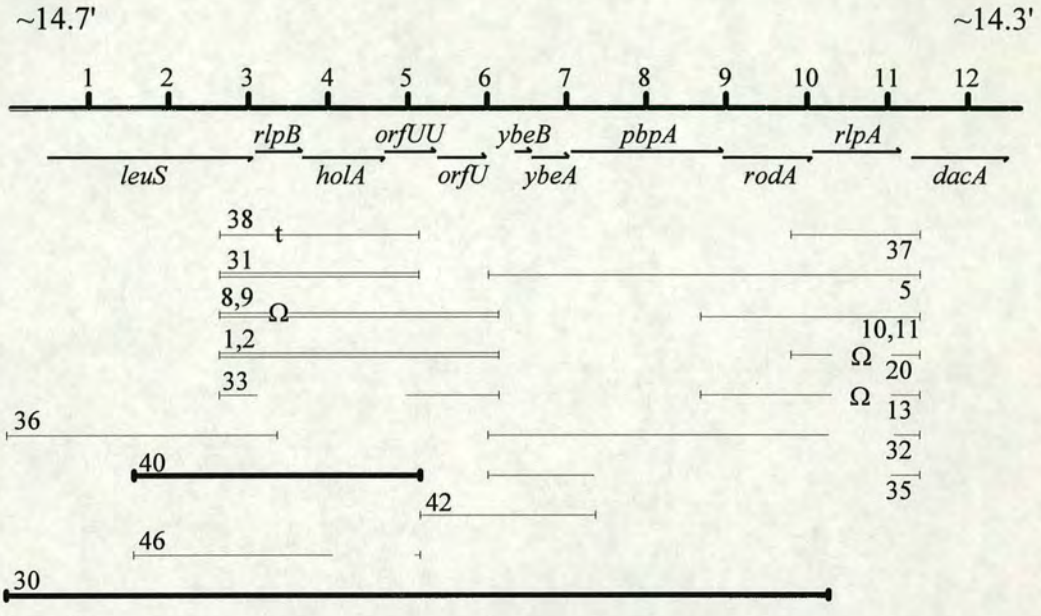


Figure 5.3.4 *Complementation results for holA mutants.* In an adaptation of Figure 5.3.1, bold lines indicate plasmids which gave good complementation of strains FI139 and 205. Those represented in double lines complemented poorly, as described in the text. Plasmids in feint lines were either not used or did not complement; see Table 5.3.3.

Table 5.3.3 Complementation results for *holA* mutants.

		Plasmid pADD:								
		2	5	8	30	31	33	38	40	46
Mutant strain:	FI139		–		+	+/–	–		+	–
	FI205	+/–		+/–	+	+/–		–	+	–
Complementation: +, yes; –, no; +/–, poor.										

Complementation of these mutants was complex. They were found to be complemented by pADD30 and then only very poorly by pADD2, 8 and 31. This poor complementation manifested itself in the formation of very small, translucent colonies containing filamentous

and lysing cells, at temperatures from 34°C to 44°C. In contrast to the *ts* mutants themselves however, there was no drop in number of colonies between 30°C and 44°C. It had been established that pADD2 and its derivatives which contained *holA*, were deleterious to cell growth; see section 5.3.1. At least some of this effect was probably due to *holA* in very high copy number. It was thought possible that the presence of some HolA was complementing the lethal mutations but that the higher than necessary levels of the protein were having the deleterious effect. The fact that the filamentous phenotype only occurred between 34°C and 42°C was consistent with the phenotype corresponding to the onset of the chromosomal *ts* mutation. Why this would give a worse phenotype in a *holA* mutant than in a wild-type strain is unclear. It is also unclear why pADD38 did not complement (even poorly). Perhaps the expression of *holA* from this construct, which has the Tet^R gene transcribed in the same orientation as *holA*, is much higher than in the others causing lethality, but only in the mutants. It remained a possibility, albeit unlikely, that this was some form of poor multicopy suppression. Plasmids pADD40 (*rlpB* plus *holA*) and pADD46 (*rlpB* only) were constructed, from much lower copy number vectors. Good complementation of the two mutants by pADD40 and not by pADD46 confirmed that both the mutations were in *holA*.

Double Mutants. Two spherical mutants gave complementation characteristics which suggested that they were double mutants. Both FI691 and 915 were spherical or very rounded rods at 30°C and lysed as large spheres at 42°C. When these strains were transformed with plasmids which complemented the *pbpA* mutants described above, they changed from spheres to rods at the permissive temperature but were still *ts*. No other plasmids available from this region gave any form of complementation to these strains. At the non permissive temperature, FI691 partially complemented with pADD5 or pADD32, was a mixture of filaments, lysing spheres and spheroplasts. This complex phenotype seemed to suggest that FI691 had a *pbpA* mutation in conjunction with an another mutation from outwith the region covered by the plasmids. FI915 showed very similar properties to FI691. Further study of FI691 showed that its *ts* and shape were

complemented by λ*supF*. This result indicated that either [i] the strain was a double amber mutant, one in *pbpA* and the other outwith the region, [ii] it had a polar amber mutation in an essential gene upstream of *pbpA*, [iii] it had a polar amber mutation in *pbpA* which caused lethal overexpression or underexpression of a downstream gene or [iv] an amber in *pbpA* suppressed at only 10% by the *supF*^{ts} in OV2 at 30°C, produced a truncated PBP2 protein 90% of the time, which was mildly antagonistic to the wild-type at this ratio giving spherical cells, but was activated at 42°C to become extremely dominant over the wild-type. Neither of the first three explanations seems particularly likely: [i] because the frequency of amber mutations was very low, [ii] because a terminator had been detected upstream of *ybeB* (Chapter 4) and all downstream DNA was in a plasmid (pADD5) which only partially complemented FI691 and [iii] because overexpression of *rodA* and *rlpA* is not lethal and overexpression of *dacA* causes cells to become spherical (the partially complemented cells were rod-shaped). The fourth explanation seems quite unusual but may be the only explanation which fits the data. Therefore the nature of the mutation in this strain (and that in FI915) remains unknown.

Mutants in uncharacterised genes. Four of the mutants obtained from this screen were found not to be complemented by any part of the region available on plasmids. That is, FI456 was not complemented by pADD30, 31, 33, 35 or 37; FI1159 was not complemented by pADD30, 5 or 37; and neither FI1623 nor FO1165 were complemented by pADD30 or 5. These were separable into two groups on the basis of their phenotype. FI456, 1159 and 1623 were very similar in phenotype to the *leuS* mutants, described above; that is, they were clean *ts* mutants which appeared to swell very slightly and get slightly longer before dying at the non permissive temperature. The dead cells appeared phase bright although they had apparently not lysed. FO1165 had a very distinct phenotype in that it was quite long at 30°C and extremely filamentous at 42°C. These four mutants, being in genes outwith the region covered by the project would not have been studied further in this thesis; however, the

possibility that FO1165 was a new cell-division mutant prompted a small amount of further characterisation.

It is possible to get wild-type recombinants of mutant strains by infecting with phages from the Kohara collection which contain the wild-type version of the mutated gene. The Kohara phages cannot lysogenise because they are cI^- but if they are spotted at high titre onto a lawn of mutant strain at the non-permissive temperature, recombination can occur between phage and chromosomal DNA to leave wild-type colonies. This is not an ideal screen because sometimes false negative results can occur, however a positive result with many colonies around and within the spot of phage, is usually correct (KJ Begg, unpublished). This experiment was carried out as described above, on each of the four unknown mutants, using Kohara phages from around the fifteen minute region (λ1G6, λ15D7, λ3A2, λ16A8 and λ3A6; see 2.1.2). It was found that both FI456 and FI1159 gave positive results with λ16A8 while neither FI1623 nor FO1165 gave colonies with any of the phage tested. λ16A8 contains a region of about 0.2' which is not in any of the other Kohara phage. This includes *glnWU*, *glnXV*, *leuW*, *metU*, *metT*, *asnB*, *nagD* and about 4 kb of unsequenced DNA. The mutations in FI456 and 1159 are unlikely to be in any of the first six of these loci as they all represent duplicate genes (Riley, 1993).

The most interesting of the four mutants, FO1165 was investigated further. Filamentation can be due to a number of different phenomena, some of which are only very distantly associated with cell division. For example: many filamentous mutants turn out to be defective in some aspect of the SOS response to DNA damage. They undergo filamentation in response to DNA damage, by inducing the production of SulA, an inhibitor of the essential cell-division protein FtsZ (Bi and Lutkenhaus, 1993); mutations in the *rpoH* gene, for the heat shock-specific sigma factor, can cause filamentation (Gibbs *et al.*, 1992); constitutive expression of enzymes involved in fatty acid oxidation (due to *fadR* mutations) causes filamentation at high growth rates (Vanderwinkel *et al.*, 1976). A prospective division mutant such as FO1165 must be shown not to be defective in these indirect modes of division inhibition (despite the knowledge that FO1165 was known to be mutated at a different

location from these genes). FO1165 showed neither the short filaments characteristic of *rpoH* mutants (Gibbs *et al.*, 1992) or the growth rate dependent filamentation of the *fadR* mutation (Vanderwinkel *et al.*, 1976), so the possibility of it being an SOS induced filamentation mutant was investigated.

If overproduction of FtsZ can suppress the filamentation, then the division block may be due to inhibition of FtsZ probably through induction of the SOS response. If FtsZ overproduction does not suppress the defect, then the strain is worth investigating further as a division mutant. With this in mind, FO1165 was transformed with pSUZ, which overexpresses *ftsZ*, and pBR322 as a control. It was found that transformants with both of the plasmids were still *ts* and filamented extensively at the non-permissive temperature. The pSUZ transformants formed smaller colonies and filamented slightly more than the pBR322 transformants, at the permissive temperature. These results enhanced the possibility that FO1165 was mutated in a new cell-division gene.

Figure 5.3.5 presents a growth curve and cell size measurements of FO1165 undergoing a step up in temperature, showing its filamentous phenotype. Figure 5.3.6 shows pictures of FO1165 at the permissive and non-permissive temperature, with nucleoid staining revealing a regular distribution of nucleoids along the filaments. This further indicated that FO1165 was not a replication or partitioning mutant in which filamentation was caused indirectly.

Figure 5.3.5 *Temperature sensitive filamentation of FO1165.* The OD_{600} of FO1165 cultures grown at 30°C (□) and 42°C (■) and the median cell volume at 30°C (○) and 42°C (●) are shown. Broth cultures were maintained in log phase for most of the experiment by keeping their OD_{600} between ~0.1 and 0.2; however, once the steady state growth of the 30°C culture was established, its growth was allowed to continue.

The OD_{600} shows that initially, both cultures grew at very similar rates. At 275 min (125 min after the temperature shift), the 42°C culture noticeably began to slow down and by 400 min it had almost stopped growing. [It is possible that the growth rate actually began to slow down as early as 200 min.]

Median cell volume data shows that while cells at 30°C did not change their volume significantly during the experiment, those at 42°C enlarged dramatically, immediately after the temperature shift-up, until about 350 min where their volume plateaued. The rate of increase in volume was not quite as fast as the OD_{600} increase until about 275 min and so it could be inferred that there was some residual cell division until that time. The filaments reached approximately 6x the volume of the cells at 30°C. The presence of lysing cells in that culture could have reduced the values obtained towards the end of the experiment, indeed the cells shown in Figure 5.3.6 appear to be more than 6x the length of the controls.

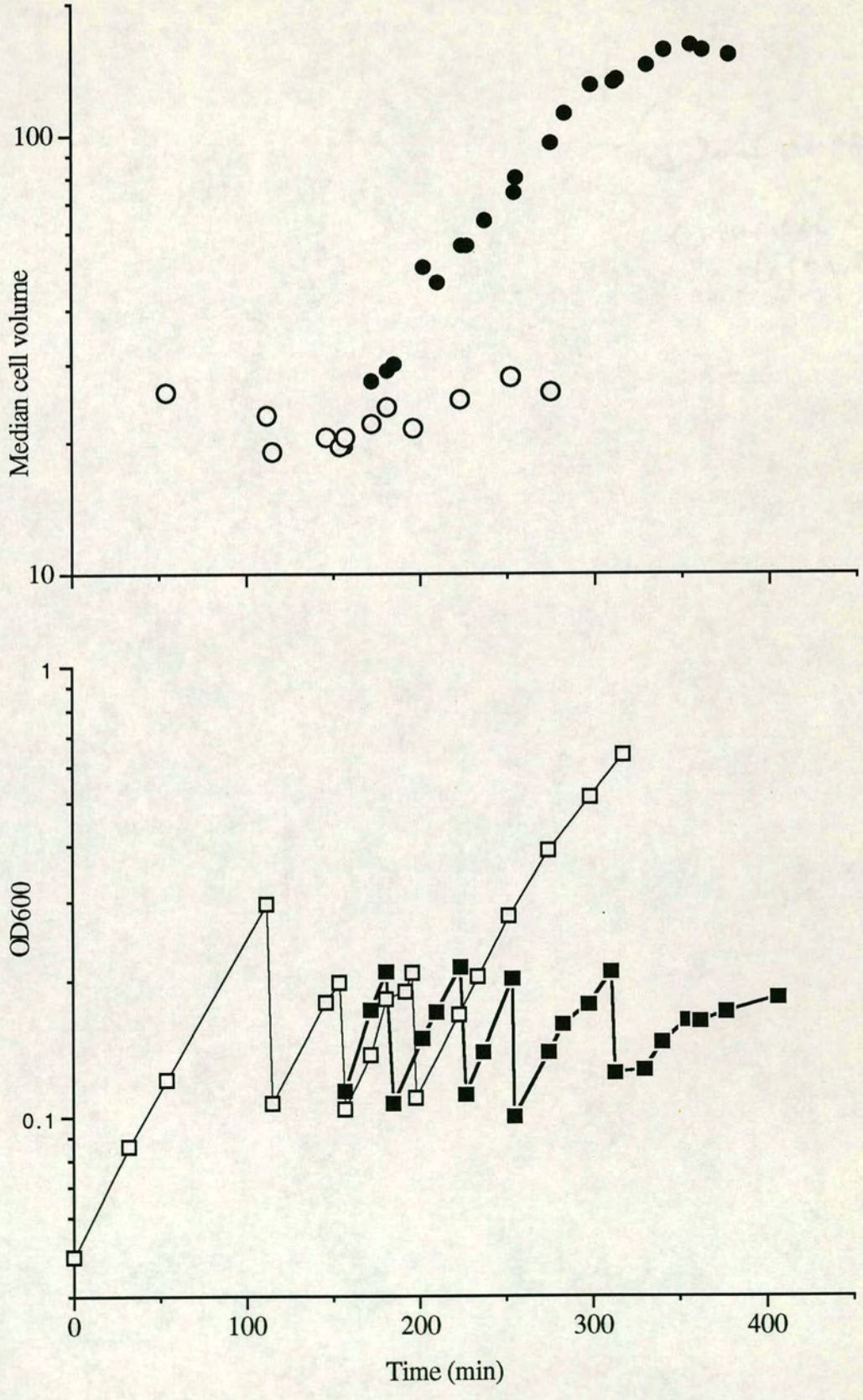
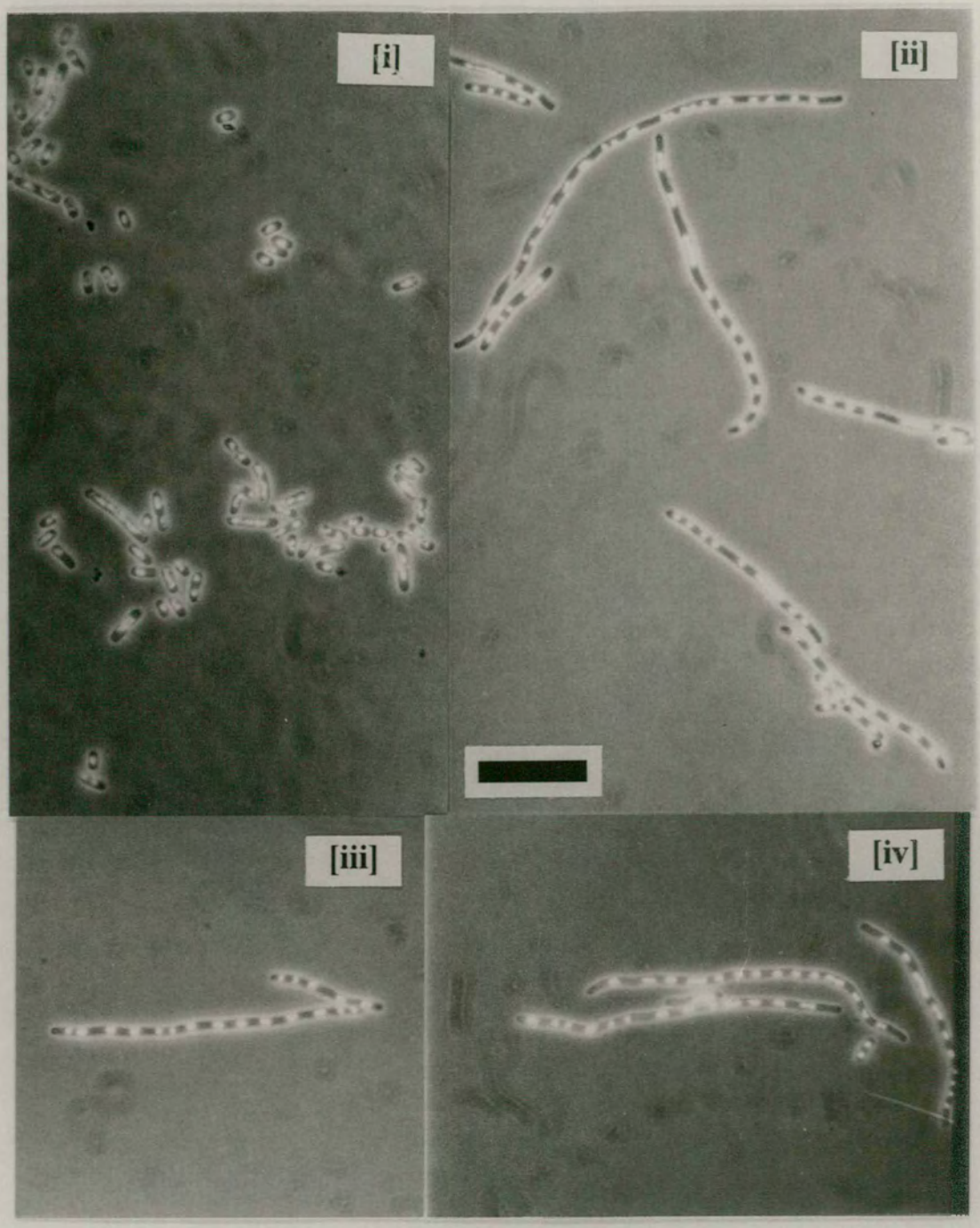

Median cell volume
100
10
OD600
1
0.1
0
100
200
300
400
Time (min)

Figure 5.3.6 *The FO1165 phenotype.* Cells from the growth curve illustrated in Figure 5.3.5 were taken for microscopic analysis using the method for visualisation of nucleoids (*2.3.5*). Samples were taken from the 30°C (154 min) and 42°C (254 min) cultures at comparable OD_{600}s.

[i] 30°C (154 min) sample [ii] – [iv] 42°C (254 min) sample. The scale bar represents 10 μm. It is very obvious that the mutation in FO1165 causes extreme filamentation at the non-permissive temperature. Also, the nucleoid distribution infers that chromosome replication and partitioning is unaffected by the mutation. As suggested in the legend to figure 5.3.5, the filaments appear to be more than 6x the length of most of the 30°C cells.


[i]
[ii]
[iii]
[iv]

Attempts were made to move this mutation into a different strain background. Phage P1 transduction (*2.4.6*) of C600 and W3110 to Tet^R was carried out with a lysate made on FO1165. As a control, OV2 was used as a recipient. All Tet^R OV2 transductants were also *ts* indicating that the mutation in FO1165 was very close to the transposon. These colonies all filamented at the non-permissive temperature (42°C). 70% of Tet^R C600 transductants were also *ts* on NBT and filamented at 42°C. None of the Tet^R W3110 transductants were *ts* at 42°C. This was a strange result. It appeared that the effect of the mutation was very much background dependent. Ten transductants from each recipient were then patched onto minimal medium (appropriately supplemented for OV2 and C600) at 30°C and 44°C. At this extreme non-permissive temperature all the transductants, irrespective of background, were *ts*, but the W3110 transductants did not filament. [Interestingly, although both C600 and W3110 grew well at 44°C, they showed slight morphological defects, including a low proportion of forked and kinked cells. OV2 did not grow well at this temperature.]

Given further time, the strain dependence of this mutation would have been investigated. It may be possible that this mutant has highlighted another indirect pathway to cell-division inhibition which acts through something other than FtsZ (perhaps FtsW, a cell division protein which seems to act at an early stage in division, like FtsZ, Khattar *et al.*, 1994). Because the position of the mutation is very close to the transposon from CAG12149, identification of the gene in which this mutation exists should not be difficult. The gene harbouring this mutation was given the name *ftsP*.

5.3.3 Discussion

A striking result of this screen was that no mutations were obtained in any of the uncharacterised genes or open reading frames in the fifteen minute region. A few explanations can be offered for this. [i] It may be an inevitable result of the position of the two transposons. Although they were the closest available on either side of the region, the transposons may still have been far enough away from the uncharacterised genes, for the lower frequency of cotransduction and smaller amount of DNA (37% of the region) to combine to give a

statistically low chance of isolating mutants in those genes. The transposon nearest to *leuS* provided five *leuS* mutants, two *holA* mutants and six *pbpA* mutants and so did not seem to be heavily biased to one side of the region. The above explanation therefore may not be valid. [ii] It is however significant that most of the detected mutants were in by far the two biggest genes (*leuS* and *pbpA*). It may therefore be the case that some or all of the other genes in the region have significantly fewer codons that can be changed by hydroxylamine mutagenesis to give a temperature sensitive protein. It is impossible to predict such amino acid changes, but codons which can give amber mutations after hydroxylamine mutagenesis are TGG and CAG. The proportion of the total number of these in the fifteen minute region which are in the uncharacterised genes (36%) is almost exactly the same as the proportion of DNA (37%) which these genes take up. There is therefore no bias towards the characterised genes in this respect at least. [iii] It is known that *dacA* is not an essential gene (Spratt, 1980) and consequently, even though one of the transposons was nearest to that gene, no *dacA* mutants were detected. If none of the uncharacterised genes are essential then this approach would not be successful.

Some hope was provided however, by the fact that no mutants were isolated in *rodA*, an important gene required for maintenance of cell shape, and the third biggest of the region. *rodA* is non-essential (Spratt, 1980, KJ Begg, unpublished) but, as was shown for FI164 and FO499, non-lethal, conditional spherical mutants were detectable using this screen. [A further conclusion which could be drawn from the data in Spratt (1980) is that all DNA between *rodA* and *lip* is non essential. This is very relevant to this thesis as *rlpA* is situated in that region. Before this conclusion was made however, the particular strain with this deletion would need to be analysed by Southern hybridisation for the *rlpA* gene.]

Luck could therefore be the overriding reason for the lack of novel mutants and screening of more transductants would probably produce some.

5.4 Attempts to Delete or Interrupt Uncharacterised Genes

5.4.1 Introduction

The notion that none of the uncharacterised genes are essential, led to a more direct approach to characterisation: by attempting to disrupt and/or delete the chromosomal copies of these genes. Various methods for gene disruption were used without success; however, one method was found which worked reasonably well. Gene disruption was attempted for *rlpB*, *orfUU*, *orfU*, *ybeA* and *rlpA*, but was only successful for *rlpB* and *orfUU*. Given further time, the method which proved to be reliable, would have been used to obtain chromosomal disruptions in the other genes.

5.4.2 Methods for chromosomal gene disruption

Most methods involved the construction of plasmids containing the gene in question, disrupted by a cassette containing a selectable marker, with enough DNA homologous to the chromosome either side of the cassette to permit recombination between plasmid and chromosomal DNA. Recombination either side of the insertional cassette would result in the chromosomal gene being disrupted by the insertional cassette. Depending on the technique employed, these two recombination events would be required to occur in one or two steps.

Linear transformation. A standard method for making chromosomal gene disruptions, used both in eukaryotes and prokaryotes, is transformation with large amounts of linear DNA from plasmids containing the gene of interest disrupted by a selectable marker. Linear transformation in *E. coli* is routinely performed in strains mutant in intracellular exonucleases such as *recD* strains. The *recD* mutation inactivates the ExoV exonuclease, a complex of proteins including RecD. The presence of such a mutation for this technique is important since linear DNA would normally be rapidly degraded after entering the cell. This method is described in detail in Russell *et al.* (1989).

Plasmids were constructed that disrupted: *rlpB* with the Ω fragment (pADD8, 9), *rlpB* with the Tet^R gene from pBR322

(pADD38), *orfUU* with the Ω fragment (pADD6, 7), *rlpA* with the Ω fragment (pADD13) and *rlpA* with the Kan^R cassette from pUC4-K (pADD21). Two different recipient strains were used in linear transformation experiments with these plasmids. DL307 has an unsuppressed amber mutation in *recD* and D301 has a mini-Tn*10* insertion in *recD*. In these experiments either, [i] the entire plasmid was linearised by restriction with a single enzyme and gel-purified or [ii] a DNA fragment containing the gene of interest disrupted by the cassette, was isolated by restriction digests and gel-purified; see Chapter 2 for methods. In both cases as much as 5 μg of the transforming fragment was prepared for each transformation. Transformation was carried out by the two methods described in Chapter 2 (*2.3.1*) although the higher efficiency of electroporation was favoured over the TSS method. Transformed cells were plated out with selection for the marker on the cassette.

If the gene in question is essential, this technique will not work unless a complementing plasmid is already present. Therefore DL307 and D301 containing plasmids which could potentially complement *rlpB*, *orfUU* and *rlpA* disruptions were also transformed in this way.

Circular transformation. Certain methods involve the use of uncut plasmids transformed into particular mutant strains in which recombination with the chromosome is selected for or enhanced.

The product of the *polA* gene, DNA polymerase I, is required for plasmid replication. Therefore transformation of *polA* strains with plasmid DNA is very inefficient. It is possible to use plasmids like those described above for transformation into *polA* strains and then select for the cassette. The only way in which the marker on the cassette can be retained by the transformant is if recombination occurs between plasmid and chromosomal DNA. If a single recombination event occurs then the entire plasmid is integrated into the chromosome; if a recombination event occurs either side of the cassette, then the disrupted gene remains on the chromosome while the wild-type gene is on the excised plasmid. This plasmid is lost from the cells very quickly because it cannot replicate. The *polA* strain ND13 was used in experiments of this type with transformations performed as before except with intact plasmid DNA.

A modification of this technique was the use of a *polA*^{ts} mutant strain (JC411), which could be transformed normally at the permissive temperature. A shift-up in temperature under appropriate selection would select for cells in which the plasmid had integrated into the chromosome. After a shift-down in temperature plasmid excision under selection would not be lethal and one could screen for the desired excision event. If this was successful, the excised plasmid, now containing the wild-type gene, would be able to complement a lethal disruption. Disadvantages associated with using *polA* mutants are that they grow poorly (this work, Joyce and Grindley, 1984) and tend to make poor competent cells.

recB, recC, sbcB, sbcC strains were originally used for linear transformation but it has been found that a similar degree of success in chromosomal replacement experiments could be attained by using circular DNA (Oden *et al.*, 1990). These mutations induce plasmids to replicate predominantly by the rolling circle mode resulting in a large amount of linear plasmid DNA in the cell, a better substrate for homologous recombination with chromosomal DNA than circular DNA. Also, the presence of large amounts of linear DNA is apparently deleterious to the cell, so if selection is applied for the cassette then there is a positive selection for cells in which the cassette has integrated into the chromosome (Oden *et al.*, 1990). A small proportion of circular transformants of these strains therefore undergo two recombination events between the plasmid and the chromosome, leaving the cassette on the chromosome. Again this technique is not suitable for disruption of an essential gene.

Phage Lambda Transduction. A method described by Kulakauskas *et al.* (1991) allows utilisation of the large pieces of chromosomal DNA in λ transducing phages. The idea is to infect a strain, containing plasmids with the appropriate disruption, with a lambda phage carrying insert DNA from the same region of the chromosome. The resulting lysate should contain some phage which have gained the disrupted gene by recombination between the plasmid and the phage. A pure lysate of these recombinant phage can be made by isolating a lysogenic colony with the resistance carried by the cassette, and then inducing this lysogen. The lysate can then be used for transduction of

the disrupted phage-borne gene to the chromosome. This final transduction can be performed on a strain containing a potentially complementing plasmid in case the disruption is lethal.

Temperature sensitive plasmids. If the disrupted gene is cloned into a plasmid which is *ts* for replication, a technique analogous to the use of *polA*^{ts} strains can be employed (Hamilton *et al.*, 1989). That is, at the non-permissive temperature the plasmid cannot replicate. If selection is applied for plasmid markers then the host cells can only survive if the plasmid is integrated into the chromosome. The plasmid could then excise; however, if there is still selection for plasmid markers, these cells will die because the plasmid cannot replicate. When cells are returned to the permissive temperature, plasmid excision is not lethal. If the homology to the chromosome is reasonably large and of similar size on either side of the disrupting cassette, there is a good probability that some excisions will leave the disruption on the chromosome. In this case complementation of a lethal disruption is automatically provided by the presence of the excised plasmid which contains the wild-type gene. An advantage of this technique is that it can be carried out in a healthy wild-type strain. Also the *ts* nature of the complementing plasmid allows the phenotype of the disruption to be observed at the non-permissive temperature (when the plasmid is lost from the population).

For this technique, plasmids were constructed that disrupted: *rlpB* with the Ω fragment (pADD19), *rlpB* with the Tet^R gene from pBR322 (pADD50, 51), and *orfUU*, *orfU* and *ybeA* with the Kan^R cassette from pUC4-K (pADD56, 57; pADD54; pADD55, respectively).

The only approach which proved successful in this work was the use of *ts* plasmids. Problems experienced with the other techniques will be discussed briefly and then the results of the use of *ts* plasmids will be described.

5.4.3 Results with unsuccessful techniques

The use of linear transformation is a very common technique and it is not clear why it did not work for these particular constructs. However, electro-competent cells prepared from DL307 and D301

cultures appeared not to transform as well as wild-type strains. All colonies obtained from transformations selecting for the cassettes turned out to contain intact plasmid which was originally linearised or cut into two fragments. Despite the DNA being cut and gel-purified twice, these circular transformant colonies still appeared in very small numbers. This was taken to indicate that the frequency of transformation was still high.

Previous use of the *recB*, *recC*, *sbcB*, *sbcC* strain DL51 in this laboratory was not successful (N McLennan, personal communication) and so this method was not used. Use of *polA*^{ts} and amber mutants was mildly successful in that it was fairly simple to isolate strains with the appropriate plasmids integrated into the chromosome. In one case it was shown by Southern analysis of chromosomal DNA that the plasmid had definitely integrated into the chromosome; however, with the *polA* amber mutants, the desired double recombination events leaving the cassette on the chromosome were never detected. In the *polA*^{ts} strains with integrated plasmids, all plasmid excisions that were detected still had the cassette on the plasmid. This may have been because the plasmids which would have been produced by the desired excision event were not sufficient to complement the disruption. P1 lysates were made on those strains with plasmids integrated into the chromosome in order to transduce the entire region into a wild-type strain. Transductants with the resistance carried by the cassette had all undergone the excision event which was not desired. Attempts to retransduce a *polA* mutant with these lysates gave no colonies at all suggesting that the plasmids often (or always) excised from the P1 DNA before it recombined with the chromosome.

In the experiments with lambda phages, λ15D7 from the Kohara collection was used to infect strains containing the plasmids described above. Recombinant phage were obtained but all had gained the entire plasmid by a single recombination event, rather than just the disrupted gene. No phage of the latter type could be isolated for any of the plasmids used in this technique.

5.4.4 Use of temperature sensitive plasmids

Methods. The temperature sensitive vector used in these experiments was the Cm^R, pMAK705 plasmid, described by Hamilton *et al.* (1989). Their procedure was followed for the seven pMAK705-based plasmids mentioned above (*5.4.2*). Briefly, a transformant of C600 containing the appropriate plasmid was grown overnight at 30°C. A dilution of this culture was plated onto LBCm such that it gave single colonies at 30°C. 100x more cells were plated on a LBCm plate at 44°C. The 30°C plate was a control and colonies which appeared on the 44°C plate were chromosomal integrants of the plasmid. Ten separate chromosomal integrants were purified twice on LBCm at 44°C and then pooled by inoculating each into the same flask of LBCm, which was then shaken at 30°C overnight. This was to allow excision of the plasmids from the chromosome. The culture was diluted 1/100 into LBCm and grown overnight twice. This culture was then plated out for single colonies on LBCm plates. These potential disruptants were screened for the presence of a plasmid which did not contain the original cassette, which would be an initial indication that disruption had been successful.

Initial failures. Unfortunately this technique did not work for *rlpB*::Ω (pADD19), *ybeA*::Kan^R (pADD55) or *orfU*::Kan^R (pADD54) constructs, however successful disruptions were made in both *rlpB* and *orfUU*; see next section. The lack of success with pADD19 may have been due to the presence of a transcriptional terminator and translational stop codons in three reading frames in the Ω fragment. If the presence of one or both of these had a polar effect on genes downstream of *rlpB*, then the plasmid containing the wild-type *rlpB* gene, which would automatically be produced after the desired excision, would not complement the disruption. It was also thought that the homology to the chromosome was not sufficient on one side of the Ω fragment in this construct. Such an imbalance could make the desired product very rare. With these two points in mind *rlpB* was cloned into pMAK705 in the centre of a larger fragment of DNA (pADD44) and then disrupted by the Tet^R gene from pBR322 (pADD50, 51). Both these constructs produced successful *rlpB*::Tet^R disruptions; see below.

The reasons why neither pADD54 or pADD55 were successful was again probably due to asymmetry of the amount of homologous DNA either side of the Kan^R gene. For each plasmid, small-scale plasmid preparations were made on 24 separate potential disruptants. For pADD54 23/24 contained the original plasmid and 1/24 seemed to have the correct plasmid for a disruption to have been successful. This strain was found to be Kan^S and so to have lost the cassette completely by some unexpected recombination event. For pADD55, 18/24 colonies contained the original plasmid and the remaining 6 had no plasmid DNA at all (thus the plasmid was still on the chromosome, since selection for the Cm^R gene on the pMAK705 backbone was always maintained). For both these plasmids a further 76 potential disruptants were analysed, this time by pooling them in groups of five for plasmid preparations, and none contained plasmids indicative of the desired excision event.

5.4.5 Disruption of rlpB

Identification of potential rlpB disruptants. Potential disruptant colonies were analysed for C600 pADD50 and pADD51 cultures, as described above for pADD55 and pADD54.[10] 19/24 and 14/24 colonies respectively, contained the plasmid expected if a disruption had occurred. These plasmids gave identical restriction patterns to pADD44, into which the cassette had been originally cloned. They were therefore called pADD44' when describing strains (*2.1.1*). One probable disruptant using each plasmid was taken for further study, whilst the others were stored.

The two strains (SHA87 and SHA88) were first checked for the presence of the cassette by plating on to LBT. They both appeared to be Tet^R but grew very poorly. [Here it was realised that the Tet^R gene from pBR322 was probably not a good choice for a chromosomal insertion cassette. Its expression is constitutive (Backman and Boyer, 1983) and thus the Tet^R it confers is proportional to copy number (Allard and Bertrand, 1993 and references therein). Despite this setback it was found that both strains grew well on plates containing

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[10] The only difference between pADD50 and pADD51, was the orientation of the Tet^R cassette (*2.1.3*).

4 μg ml^{-1} tetracycline rather than the usual 10 μg ml^{-1}.] The fact that the strains were still Tet^R but that the cassette was no longer on the plasmid indicated strongly that the chromosomal *rlpB* gene had been disrupted in both cases, with the transcription of the Tet^R gene in the same direction as *rlpB* for SHA87 and the reverse for SHA88.

Analysis of temperature sensitivity and attempts to cure strains of complementary plasmids. In order to see if this disruption had any effect on the cells, the two strains were checked for a *ts* phenotype. If the disruption was lethal, then the strains would be *ts* in the absence of selection because of the disappearance of the complementing plasmid. If the disruption was not lethal then growth at 44°C would cause loss of the plasmid and leave a pure *rlpB*::Tet^R strain. Table 5.4.1 shows the results of plating 200 μl of a 10^{-7} dilution of a 30°C overnight culture of each strain onto LB plates at 30°C and 44°C.

Table 5.4.1 Temperature sensitivity of potential *rlpB*::Tet^R strains, on LB.

		30°C	44°C
Strain:	SHA87	>150	1
	SHA88	>200	6
		No. of colonies:	

This showed that both strains were *ts* and therefore indicated that disruption of *rlpB* was lethal. Although the number of colonies on the 44°C plate was slightly higher than when the initial plasmid was integrated into the chromosome, it was most likely that these colonies were re-integrants of the plasmid. That this allowed them to survive was another indication that the disruption was lethal.

In a similar experiment, a colony of each strain was inoculated into LB and incubated at 44°C overnight. The resulting culture was plated out for single colonies and then these were tested for loss of the plasmid, by screening for Cm^R. Loss of the plasmid but maintenance of the Tet^R would indicate a non-lethal disruption. Overnight cultures of both SHA87 and SHA88 at 44°C grew extremely

poorly, and single colonies from the cultures were all Cm^R. When the experiment was repeated with one of these colonies, then Cm^S colonies were detected but these were also Tet^S. This was taken to mean that it was not possible to cure the strains of the complementing plasmid, unless it integrated into the chromosome, and came back out again having regained the cassette, leaving a wild-type *rlpB* gene on the chromosome. This also suggests that disruption of *rlpB* is lethal.

It is worth repeating that both these experiments were carried out using LB without selection. Thus, the only difference between the strains at 30°C and 44°C was that at the higher temperature the plasmids could not replicate. Temperature sensitivity was therefore an indication that a strain's viability was totally dependent on the presence of the plasmid.

Molecular confirmation of the disruption. It was important to prove that the Tet^R gene was in fact on the chromosome of SHA87 and SHA88 and that it was definitely in *rlpB*. Large-scale chromosomal DNA preparations (*2.2.12*) of both strains were used for Southern analysis. Because of the obligatory presence of a complementing plasmid, restrictions were carefully designed so that chromosomal bands could be distinguished from brighter plasmid bands. Two approaches were taken; [i] to cut either side of the *rlpB* gene with an enzyme that did not cut in the cassette, so that the disrupted gene would give a larger band than the wild-type, [ii] to use an enzyme which cuts in the cassette but not in the *rlpB* gene so that the disrupted gene would give two bands where the wild-type would give one. The digests would be probed with a fragment of DNA containing only the *rlpB* gene, obtained by PCR.

Figure 5.4.1 shows the predicted Southern results for strains with the cassette either on the chromosome or on the plasmid. Figure 5.4.2 shows the actual results of this Southern analysis. Controls in these experiments were: pADD44 cut with the same enzymes as the chromosomal DNA, to give the band expected from the complementary plasmid (pADD44') and C600 chromosomal DNA as a wild-type control.

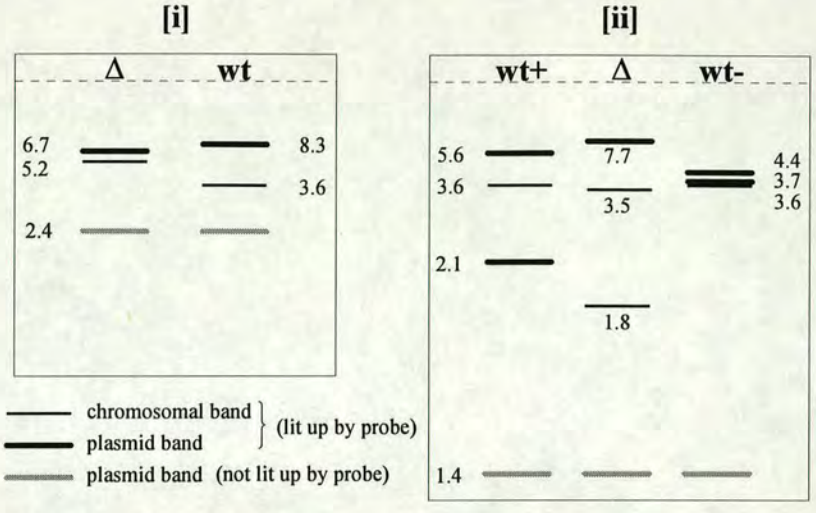
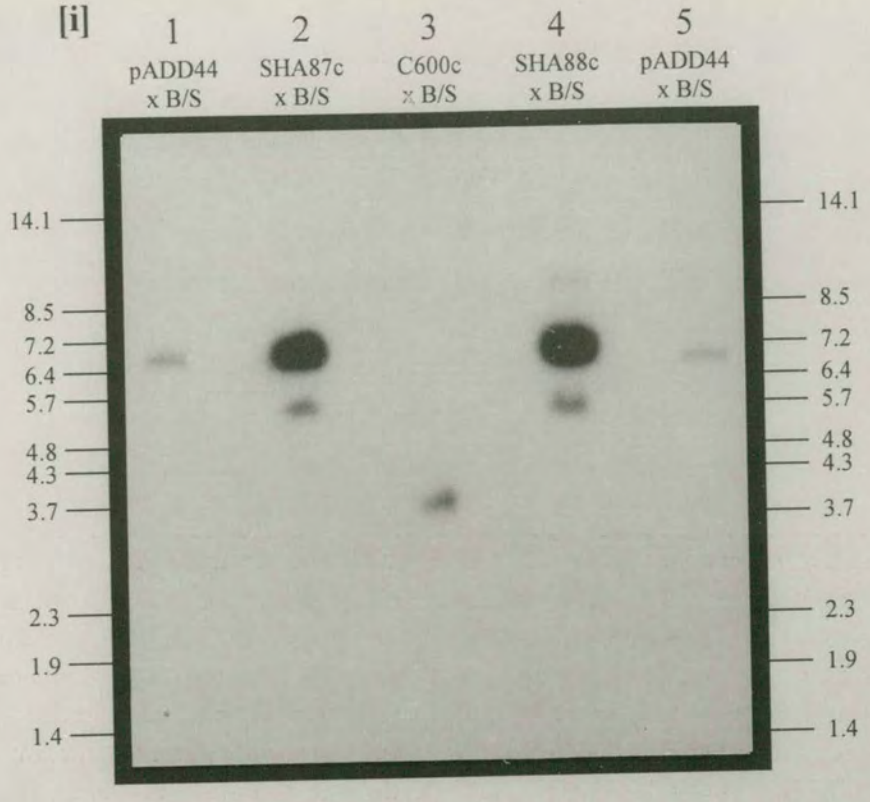
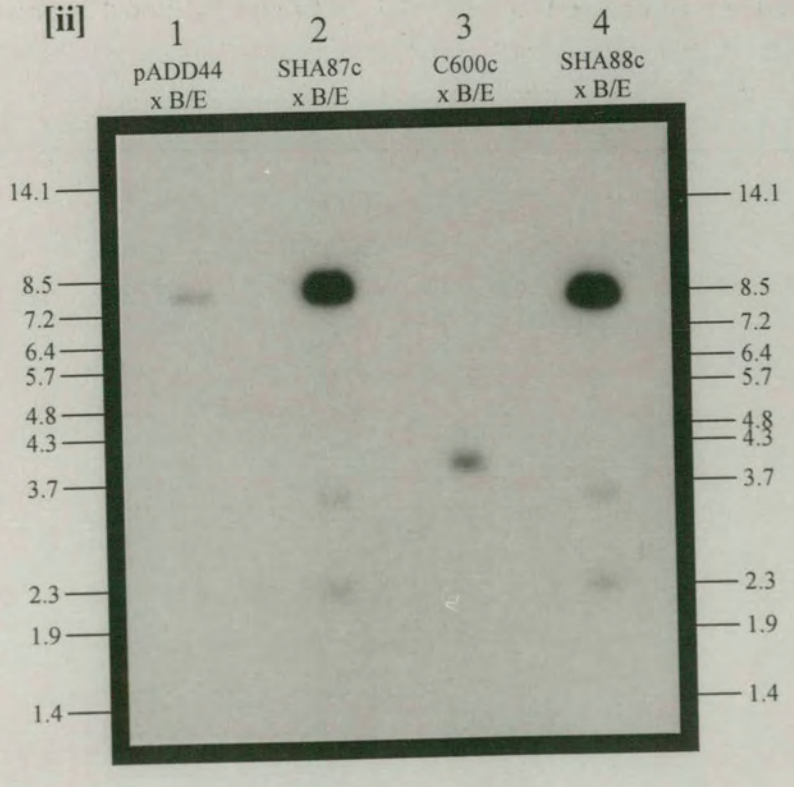


Figure 5.4.1 *Predicted band sizes for Southern analysis of SHA87 and SHA88.* A diagram of the predicted result of Southern analysis of the two *rlpB*::Tet^R strains is presented. Horizontal lines represent distinct DNA bands from an agarose gel. Black lines are those predicted to hybridise to the *rlpB* probe. Grey lines are those plasmid bands which would not hybridise to the probe. It was anticipated that chromosomal and plasmid bands could be distinguished by their intensity (the copy number of the plasmid bands being significantly higher) and this is reflected in the thickness of the lines. The two types of restriction described in the text are represented: [i] is cut with *Bgl*II and *Sma*I to show an increase in the chromosomal band containing the cassette, [ii] is cut with *Bgl*II and *Eco*RI to show that cutting with an enzyme internal to the cassette gives two bands for the chromosomal DNA containing the cassette; only if the cassette is in the *rlpB* gene itself, will both these bands light up with an *rlpB* probe. The predicted pattern for a chromosomal *rlpB* disruptant with a complementing plasmid, pADD44' (Δ) is shown alongside that of a wild-type strain containing the original plasmid, pADD50 (**wt+**) or pADD51 (**wt-**), with + or - indicating the direction of transcription of the Tet^R gene with respect to *rlpB*. In [i] the wild-type pattern would be identical whether pADD50 or pADD51 was present, likewise in [ii] the disruptant pattern would be independent of the orientation of the cassette.

Figure 5.4.2 *Southern analysis of SHA87 and SHA88.* Restriction digests, agarose gel electrophoresis, radioactive labelling of probes, Southern blotting and hybridisation were all performed as described in Chapter 2. Filters were probed with PCR-amplified *rlpB* (*2.2.8*) to detect bands containing *rlpB* sequence, and then with lambda DNA to detect the λcI^{857} x *Bst*EII markers. The latter are shown at the sides of each picture. Digests are labelled as follows: c = chromosomal DNA, B = *Bgl*II, S = *Sma*I, E = *Eco*RI. Experiments [i] and [ii] are as described in the text and Figure 5.4.1. The two experiments show that both SHA87 and SHA88 have a *Bgl*II fragment containing *rlpB* which [i] is bigger than the wild-type fragment by the size of the Tet^R gene (~1.7 kb) and [ii] is cut by *Eco*RI (a site present in the Tet^R gene but absent in the wild-type fragment) internal to *rlpB*. All plasmid and chromosomal bands are therefore as predicted for disruptants in Figure 5.4.1 and not as predicted for C600 containing the original plasmids.


[i]
1
pADD44
x B/S
2
SHA87c
x B/S
3
C600c
x B/S
4
SHA88c
x B/S
5
pADD44
x B/S
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4


[ii]
1
pADD44
x B/E
2
SHA87c
x B/E
3
C600c
x B/E
4
SHA88c
x B/E
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4

These two Southern hybridisation experiments gave proof that the *rlpB* gene is disrupted on the chromosome of both SHA87 and SHA88. The genetic experiments indicate that this disruption is lethal without a complementary plasmid, indicating that *rlpB* is an essential gene. Subsequent experiments were designed to confirm these conclusions and to observe the phenotype of an *rlpB* null strain.

F*urther proof of the lethality of rlpB null strains.* No difference had been noticed between the two *rlpB* null strains as yet and so strain SHA87 was chosen for most of the following experiments. A phage P1 lysate was made on this strain and then used for transduction experiments. Recipient strains were W3110 and W3110 pADD30 (pADD30 contains *rlpB, 2.1.3*). These were transduced to Tet^R at 30°C on 4 μg ml^{-1} LBT plates (due to the low level of tetracycline resistance provided by a single copy pBR322 Tet^R gene).

W3110 generally grew better than W3110 pADD30 and so usually gave more colonies in transduction experiments. It was found however, that with this lysate, W3110 was very difficult to transduce to Tet^R, in fact only one colony was obtained. This colony was subsequently found to be Tet^R, Cm^R, therefore resulting from transduction of chromosomal DNA containing the integrated complementing plasmid along with the Tet^R gene in *rlpB*. The original disruption experiments during which chromosomally integrated plasmids were selected for, by plating at 44°C, showed that the proportion of cells in the population with integrated plasmids was approximately 1/1500. The fact that the only Tet^R W3110 transductant had inherited DNA from one of these rare cells was a good indication that disruption of *rlpB* in W3110 was lethal.

Transduction into W3110 pADD30 gave Tet^R, Cm^S colonies of two distinct sizes. When these colonies were screened on LBT plates containing 4 μg ml^{-1} and 10 μg ml^{-1} tetracycline, large colonies grew well on both, but small colonies only grew well on the lower concentration. Small-scale plasmid preparations showed that larger colonies contained a plasmid which was not pADD30 but a derivative with the Tet^R gene in *rlpB* (pADD30*rlpB*::Tet^R). This could have arisen either from recombination between pADD30 and incoming transducing DNA, or transduction of the disruption into the

chromosome and then recombination between the plasmid and the chromosome. Small colonies still contained pADD30 and so the *rlpB*::Tet^R had been transduced into the chromosome. Therefore, the apparently unfortunate choice of cassette was actually an advantage, in that it allowed further confirmation of successful transduction of the disruption by giving a distinction between small colonies and large colonies containing single and multiple copies of the Tet^R cassette respectively. One of the small colonies, SHA89, was subsequently transduced to *recA*::Cm^R from a P1 lysate made on SHA60, to give a stable *rlpB* disruptant (in case exchange of the cassette from the chromosome to the plasmid was a frequent event). This strain (SHA90) was checked for the presence of pADD30 and then stored.

The fact that the chromosomal disruption of *rlpB* could not be transduced into W3110 unless a wild-type copy of the *rlpB* gene was also present (either in the transducing DNA or on pADD30) confirmed that *rlpB* was an essential gene, not only at 44°C, as suggested in previous experiments, but also at 30°C.

Disruption of rlpB causes a block to cell-division. Preliminary temperature shift experiments were carried out on both SHA87 and SHA88. Overnight 30°C cultures of each strain were diluted 1/100 into LB at 30°C and their OD_{600} was monitored. Once steady growth was attained, these cultures were used to inoculate fresh LB at 30°C or 44°C. Cultures at 30°C grew steadily for over 400 min when they were regularly diluted to maintain logarithmic growth. Cultures grown at 44°C gradually slowed until about 200 min where growth stopped completely. After a lag of approximately 150 min they started to grow again, eventually reaching their original growth rate. As both strains gave identical results in this preliminary experiment, SHA87 was used for an identical experiment where cell size and morphology were also monitored. Figure 5.4.3 presents these results. It shows the same features described above, together with cell-size data from Coulter channelyser analysis. The results are described in detail in the accompanying legend.

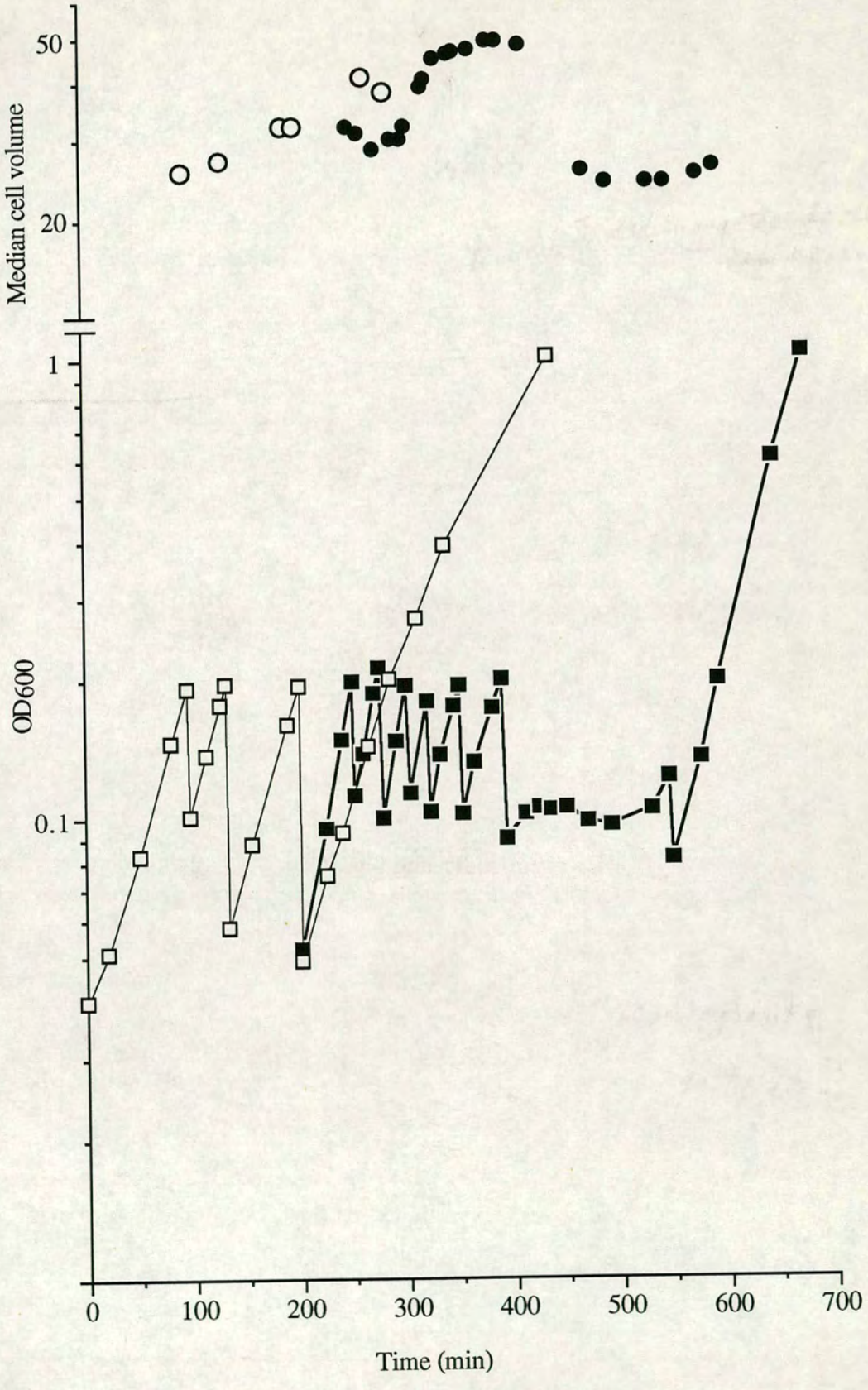
Figure 5.4.3 *Temperature shift experiment with SHA87.* The OD_{600} of SHA87 cultures grown at 30°C (□) and 44°C (■) and the median cell volume at 30°C (○) and 44°C (●) are shown. Broth cultures were maintained in log phase for most of the experiment by keeping their OD_{600} between ~ 0.1 and 0.2; however, once the steady growth of the 30°C culture was established its growth was allowed to continue.

The OD_{600} data shows that the cells inoculated at 30°C grew at a steady rate throughout the experiment. The culture inoculated at 44°C, started with a faster growth rate than at 30°C, then gradually slowed down until it stopped growing completely. After a period of stasis, the culture started to grow again, eventually reaching its original growth rate. As the experiment was carried out in LB with no selection present, the only effect of the high temperature was the gradual loss of the complementing plasmid. The phenotype was therefore due to lack of, RlpB.

The cell volume data shows that at 30°C cells got larger throughout the experiment. This is an indication that perhaps the complementation was not perfect. Cells at 44°C were initially similar in size to those at 30°C. Concomitant with the slowing of growth rate, the cells quickly increased in size reaching a peak size just before their growth stopped completely. Cell volume was impossible to estimate for a few time points and then it could be seen to fall quickly to below its original value as the growth of the culture started up again. The difficulty in estimating the volume from some cell samples was due to large amounts of lysis in the 44°C culture. This almost certainly caused the values for samples either side of the gap to be underestimated. It is evident however, that there must have been some residual division of the large cells to have maintained growth while the median volume levelled off.

The resurgence of the culture at 44°C was due to accumulation and unhindered growth of the small proportion of the population with plasmids integrated into their chromosomes. These cells have wild-type *rlpB* and therefore grow normally and are normal sized. They were slightly smaller than the original population at 44°C, again indicating that the complementation was not perfect. These remaining cells were found to be predominantly Tet^S, Cm^S, and lacked extra-chromosomal DNA, while cells from the 30°C culture were Tet^R, Cm^R and still contained pADD44' after the same amount of time. The 44°C cells had therefore originally survived because the plasmid had integrated into the chromosome and when it excised, only those cells where the wild-type *rlpB* gene was left on the chromosome survived, due to loss of the excised plasmid at its non-permissive temperature for replication.

Microscopic analysis of all the samples taken for Coulter analysis showed that the cells at 44°C got gradually bigger by filamentation, that is they elongated rather than enlarging generally. This elongation was of a very distinctive nature in that most cells had many septa of various depths along the filament, resulting in many chain-like groups of cells. They appeared to be significantly longer than the cells at 30°C supporting the suggestion that a large amount of lysis had affected the Coulter channelyser analysis. The *rlpB* null phenotype is described in more detail and illustrated in the text.


Median cell volume
50
20
OD600
1
0.1
0
100
200
300
400
500
600
700
Time (min)

It was concluded that the major phenotype of a disruption in *rlpB* was increase in cell-size due to inhibition of division and ultimate cell-death by lysis. This increase in size was significant but not as dramatic as for the *ftsP* mutation described earlier (*Figure 5.3.5*).

Dynamics of rlpB null phenotype. In an attempt to further characterise the progression of the filamentation and lysis of SHA87 cells at the non-permissive temperature, fixed samples from the growth curve shown in Figure 5.4.3 were observed microscopically and scored for the presence of normal cells, filaments and lysed or lysing cells. It was hoped that this would eliminate the remote possibility that the cells predominant towards the end of the experiment were simply from recovering filaments which divided. This might happen if the disruption was not lethal or if extragenic suppressor mutations accumulated rapidly. Filaments were loosely defined as those cells (unconstricted or in chains) which were longer than 3x the length of the smallest cells in the initial log-phase population. Smaller cells than these were deemed to be 'normal'. Lysed or lysing cells were defined as any cell showing signs of lysis, regardless of size or shape (although the latter was noted). Indications used to identify lysis were [i] cytoplasm in the process of leaking from cells, [ii] spheroplasts and [iii] ghosts. Figure 5.4.4 shows the proportion of each class of cells at nine time points during growth (*Figure 5.4.3*), from when cells reached a peak size to when small cured cells began to predominate (thus including the period of stasis).

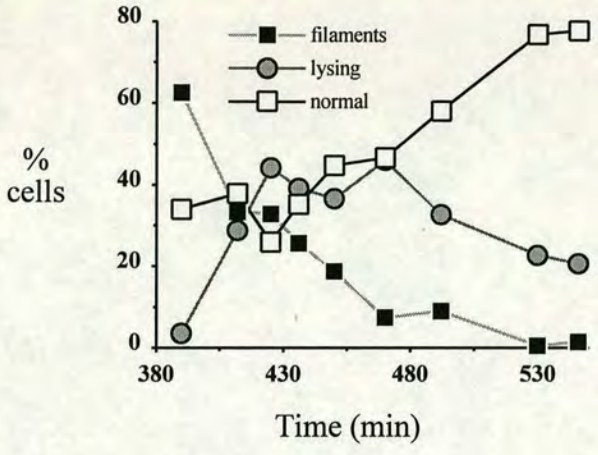


Figure 5.4.4 *Proportions of cell types during SHA87 growth curve at 44°C.* Cells from each sample were separated into three classes as described in the text. The number of cells observed for each of the nine samples were 168, 87, 170, 125, 134, 150, 165, 198 and 189. The percentage of each class in a sample is plotted against the Y-axis. The time (in minutes) at which samples were taken in the experiment described in Figure 5.4.3 is plotted against the X-axis.

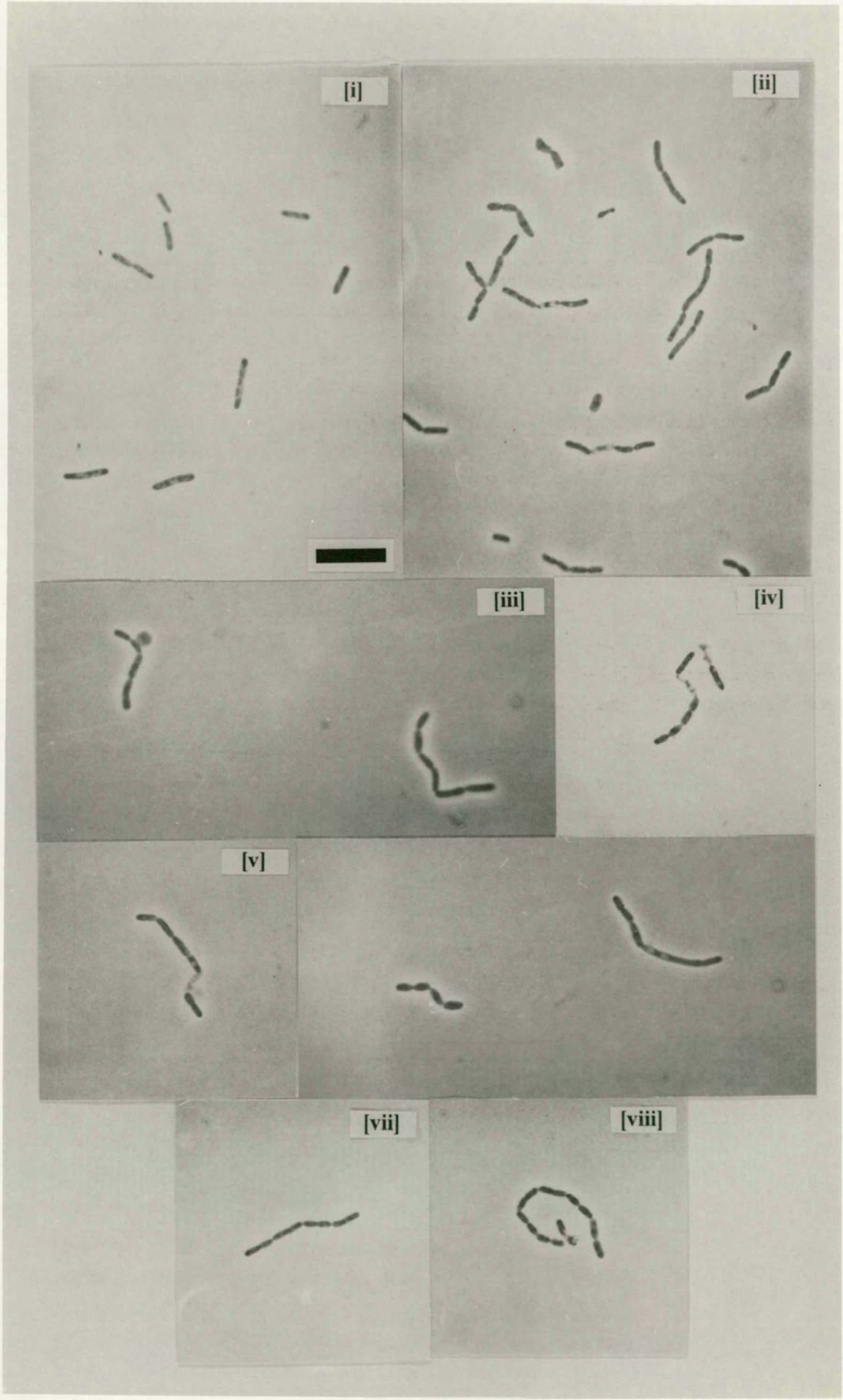
It was noted that most (but not all) lysing cells were filamentous rather than normal-sized. An interesting feature of the chains (which predominated greatly over unconstricted filaments) was that one segment of the chain could be lysing while the rest of the cell looked healthy (see next paragraph). These cells were classed as lysing cells in Figure 5.4.4. Figure 5.4.4 indicates that microscopic analysis of cells supported previous assertions about the lethality of *rlpB* disruptions. At the earliest time points, the large percentage of filamentous cells dropped rapidly, concomitant with a proportional increase in the number of lysing cells. The proportion of normal cells remained approximately constant. This suggested that, initially, filaments were undergoing lysis and thus dying from the lack of RlpB. The proportion of normal cells started to rise rapidly from about 470 min. while the lysing cells levelled off and the filamentous cells continued to fall to near zero. This could be interpreted in many ways, but is consistent with filaments continuing to lyse, and lysing cells gradually becoming debris, while the population of chromosomal

plasmid integrants and cured cells dominates the culture. The proposed residual division of filaments would mostly have taken place before these samples. If filaments were recovering due to suppressors then there would be no need to lose the chromosomal disruption, as clearly happens in these normal cells (*Figure 5.4.3, legend*). Furthermore, if the disruption was not lethal, one would not expect the high proportion of lysing or lysed cells.

Further characterisation of the rlpB null phenotype. Figure 5.4.5 shows photographs of cells from Coulter count samples of the growth curve in Figure 5.4.3. These illustrate the chain-forming, filamentous *rlpB* null phenotype and in some cases show one segment of a chain lysing without affecting adjacent segments ([iii], [v]). It can be seen that although individual segments were of approximately constant length in any one sample, the depths of septa were very variable, even within a single cell (for example [vii], [viii]). Therefore, in some segments the septa are formed well enough to osmotically isolate one from another, but this did not happen in all cases. This suggested that loss of RlpB may not stop septation completely, but it simply slows down the septation process. It is possible, therefore, that RlpB is involved in a general process which has an indirect effect on septation. A second interpretation could be that RlpB is a stable, abundant protein required for septal formation but not for lateral elongation. When the plasmid is lost, the concentration of RlpB drops slowly and septation slows concomitantly, while other processes such as initiation of septation and elongation are unaffected. Eventually there is no RlpB left and cells stop septating and quickly lyse. The presence of some apparently unconstricted filaments, similar in size to the chains, indicated that sometimes the septation was halted at an early stage. It was difficult to say whether lysis only happened at initiated septa, although the pictures in Figure 5.4.3 are not inconsistent with this idea, which might be expected if RlpB's sole function was in septum synthesis.

Figure 5.4.5 *The RlpB null phenotype.* Samples of SHA87 fixed for the Coulter counter analysis (*2.3.4*) displayed in Figure 5.4.3, were also photographed (*2.3.5*). The scale bar represents 10 μm. Plates are numbered [i] to [viii] from the top left. Picture [i] shows cells from the 30°C culture at 270 min. Pictures [ii] to [viii] show cells from the 44°C culture. [ii] = 415 min, the point immediately before the cell volumes became impossible to estimate; [iii] and [iv] = 450 min, during the time where cell volumes were impossible to estimate; [v] to [viii] = 460 min, when cell volume could be estimated again and in the period of apparent stasis. Picture [ii] is representative of the mixed population of cells in the mutant culture. As cultures became very sparse towards the end of the experiment, pictures [iii] to [viii] represent cells chosen to illustrate the features described below and in the text.

Comparing pictures [i] and [ii], both of which are from cultures of an OD_{600} of ~0.2, shows the larger size of the 44°C cells. It also shows a distinct difference in morphology; cells in [ii] are predominantly chained. One feature of [ii] is that some segments of chains have aberrantly shaped poles. As mentioned in the section about *ftsP* (*5.3.2, Mutants in uncharacterised genes.*), this is a feature of the C600 strain at 44°C and not the *rlpB* null mutation. Also relevant in [ii] is the cell with four segments in the lower middle of the picture. It shows one segment apparently lysed, however this was caused by the segment drying up on the slide. Such cells were ignored in the data presented in Figure 5.4.4. The same can be seen in the longer cell in [iv]. Pictures [iii] and [v] illustrate where one segment of a chain can lyse without appearing to affect the other segments. It is apparent in some cases, e.g. picture [vii], that some septa have progressed further than others. Pictures [ii], [iv] and [vii] show the regular distribution of DNA which was subsequently confirmed by specific staining. Cells in [ii] and [vi] show the occasional production of an aseptate filament (albeit in these cases as part of a chain).


[i]
[ii]
[iii]
[iv]
[v]
[vii]
[viii]

Unfixed samples taken from varous time points during growth (*Figure 5.4.3*) were observed under conditions allowing visualisation of nucleoids (*2.3.5*). No anucleate cells were observed and nucleoid positioning appeared to be normal in the chains. [This can actually be seen Figure 5.4.5 without specific staining or condensing of DNA, the paler regions being the chromosomes.] The usual conclusion from this is that chromosome replication and partitioning are normal. It was interesting however, that a minor proportion of unconstricted filaments contained large unsegregated DNA masses. It appeared that, in these cells, nucleoid segregation might be dependent on initiation of septum formation as suggested by Tétart *et al.* (1992). Conversely, it is possible that the formation of septa in these cells was contingent on the positioning of nucleoids and the size of spaces between them, as proposed by Mulder and Woldringh (1989). The latter is supported by the two aseptate filamentous segments illustrated in Figure 5.4.5 (*[ii]*, *[vi]*) which appear to have separate nucleoids. A very minor aspect of the *rlpB* null phenotype could therefore be the occasional failure of partitioning, leading to the formation of unconstricted filaments.

Comparisons with known chain-forming mutants. Four previously characterised mutants also cause cells to form chains. [i] The *envA* gene is essential (Sullivan and Donachie, 1984, Beall and Lutkenhaus, 1987) and is situated distal to an important cluster of cell-division and cell wall synthesis genes in the two minute region; see Chapter 1. The only known mutant in *envA* (*envA1*) is unconditional and has a pleiotropic phenotype including the formation of chains when grown in rich media (Normark, 1970, Normark *et al.*, 1969). [ii], [iii] The *lkyD* mutant of *S. typhimurium* (Chakraborti *et al.*, 1986) and the *cha* mutant of *E. coli* (Donachie *et al.*, 1984) both characteristically form chains at the non permissive temperature. [iv] The *ftsE1181*^{ts} mutation has also been shown to produce chains after prolonged incubation at the non-permissive temperature (Taschner *et al.*, 1988). *ftsE* is thought to be involved in the transport of membrane bound division proteins; see Chapter 1.

A common feature of *envA*, *lkyD* and *cha* mutants is an increased permeability to some antibiotics (Normark, 1970,

Chakraborti *et al.*, 1986). The chaining phenotype of *envA1* strains is thought to be due to a significant slowing of the process which separates two cells after the septum has completely formed. That is, two separate cells are covalently attached to one another at the poles and the step in which EnvA is involved breaks that connection (WD Donachie, personal communication). This idea comes from two observations, [i] chained sacculi can be prepared from *envA1* strains by boiling cells in SDS, proving a covalent linkage between cells (Wolf-Watz and Normark, 1976), [ii] the chaining phenotype only occurs when cells are growing fast, suggesting a slowing of the process rather than a complete inability to separate cells.

The *rlpB* null phenotype was compared with available data on these mutants (particularly *envA1* and *cha* which had been studied in this laboratory). Photographs of *ftsE1181* chains (Taschner *et al.*, 1988) appeared to be shorter, more rounded and have more blunt constrictions than the SHA87 chains. SHA87 chains were similar to *envA* and *cha* mutants, however *cha* appeared to produce much shorter segments than the former two. Despite this difference and the fact that *cha* maps to a different region of the chromosome than *envA* (*Chapter 1*), it has been shown that the *envA1* phenotype and lethality of the *cha* mutant (but not the chain forming) are complemented by a single copy of the *envA* gene on a lambda phage - λ16-2 (KJ Begg, unpublished). In order to see if the *rlpB* null phenotype was related to these mutants, SHA87 and SHA88 were lysogenised with λ16-2 and tested for temperature sensitivity; both strains remained *ts* and formed chains at the non-permissive temperature. This indication of a significant difference between the RlpB and EnvA/*cha* defects is supported by the fact that many of the *rlpB*::Tet^R cells had incomplete septa.

Therefore SHA87, although similar to previously characterised chain-forming mutants, probably has a different defect. Given further time, other features of the characterised mutants could have been looked for in *rlpB* null mutants. For example: *cha* and *lkyD* mutants were shown to have aberrant outer-membrane invagination at the septa (Chakraborti *et al.*, 1986); this could be looked for in *rlpB* null strains. If, as suggested in the legend to Figure 5.4.3, complementation of the *rlpB* disruption by the *ts* plasmid is not

perfect, then tests for permeability defects, as shown by *envA*, *lkyD* and *cha* mutants could be performed at 30°C. The activity of the EnvA protein has been found to be involved in early stages of cell wall metabolism (Young *et al.*, 1994, see Chapter 1) and its activity is known. An increase in similar activity could be looked for in RlpB-overproducing cells or preparations of purified RlpB.

Conclusions. It would be premature to say that RlpB is required for division but, it can be said that depletion of RlpB has severe effects on the normal progression of septation, with no apparent effect on cell elongation. It can also be said that the *rlpB* null phenotype is unique. RlpB is a lipoprotein with typical lipoprotein signal-sequence for external transport. It is probably located in the periplasm and a role in peptidoglycan synthesis or modification, or in cell division itself would not be surprising. This has previously been proposed by Takase *et al.* (1987). That similar chain-forming mutants seem to have minor roles in peptidoglycan synthesis is also encouraging. Further characterisation of the *rlpB* disrupted strains in conjunction with biochemical and molecular-biological experiments on the RlpB protein itself, should allow the true role of this gene to be elucidated.

It should be noted that the plasmids used to construct the *rlpB* disruptants, pADD50/51 and the resulting complementary plasmid pADD44', all contained the entire *holA* gene. This was retained originally to simplify cloning and to ensure symmetrical amounts of DNA homologous to the chromosome flanking the cassette. The presence of *holA* does however give rise to the possibility that the phenotype observed at the non-permissive temperature is due partly to a polar effect of the *rlpB* disruption on *holA* expression. Such a defect could be complemented by pADD44'. There are two good reasons to doubt this explanation: [i] *holA* mutants FI139 and FI205 were complemented (albeit poorly) by plasmids with the Ω fragment in *rlpB*. This could be a very polar insertion because the Ω fragment has stop codons in all three reading frames and a transcriptional terminator and can have polar effects in either orientation (Prentki and Krisch, 1984) [ii] SHA87, which has the constitutive Tet^R gene in the same orientation as *rlpB* and *holA*, was almost identical to SHA88, which has the same fragment in the reverse orientation, at

the non-permissive temperature. (SHA88 was very slightly more ts and poorer-growing on LBT plates than SHA87, indicating chromosomal interference with expression of the Tet^R gene rather than *vice versa*.)

Nevertheless, the presence of *holA* should be shown to be unnecessary for viability of the *rlpB* disruption. Given further time comparisons would have been made between the phenotypes of transductants for *rlpB*::Tet^R in cells containing either pADD44 or a derivative which does not contain *holA*.

5.4.6 Disruption of orfUU

Most of the work involved in the construction and characterisation of disruptants of *orfUU* followed the same procedures described for *rlpB*.

Identification of potential orfUU disruptants. Potential disruptant colonies were analysed from C600 pADD56 and pADD57 cultures, as described previously (*5.4.5*, *5.4.6*). 3/13 and 4/13 colonies respectively, contained the plasmid expected if a disruption had occurred (pADD23'). One probable disruptant using each plasmid was taken for further study, whilst others were stored.

SHA103 and SHA99 were Kan^R but the cassette was no longer on the plasmid, indicating that the chromosomal *orfUU* open reading frame had been disrupted in both cases. The transcription of the Kan^R gene was in the same direction as *orfUU* for SHA103 and in the reverse orientation for SHA99.

Analysis of temperature sensitivity and attempts to cure strains of complementary plasmids. Streaking of SHA103 and SHA99 on LB at 30°C and 44°C suggested that both strains were *ts*. Overnight cultures grown at 30°C were used to make P1 lysates and for plating out for single colonies. Table 5.4.2 shows the result of plating 200 µl of a 10^{-5} dilution of each culture onto LB at the two temperatures.

Table 5.4.2 Temperature sensitivity of potential *orfUU*::Kan^R strains, on LB.

		30°C	44°C
Strain:	SHA103	>500	>500
	SHA99	>500	31
		No. of colonies:	

This indicated that SHA103 might not be *ts*, however 25/25 of the colonies at 44°C were found to be Cm^S, Kan^S whereas those at 30°C were all Cm^R, Kan^R, suggesting that the colony used to inoculate the 30°C culture may have been a chromosomal integrant of the plasmid. Similarly, a 44°C LB culture of SHA103 grew well but was found to contain 100% Cm^S, Kan^S cells - hence all surviving cells had lost both the plasmid and the cassette.

The temperature sensitivity of SHA99 (*Table 5.4.2*), implies that disruption of *orfUU* is lethal. 22/25 of the colonies at 44°C were found to be Cm^R, Kan^R and the other 3/25 were Cm^S, Kan^S, showing that survival at the non-permissive temperature was possible only if the plasmid had integrated or if the chromosomal disruption was removed. A 44°C LB culture of SHA99 grew poorly and surviving cells were found to have retained Cm^R as well as Kan^R - indicating that the plasmid had integrated into the chromosome. Both of these results were consistent with disruption of *orfUU* being lethal.

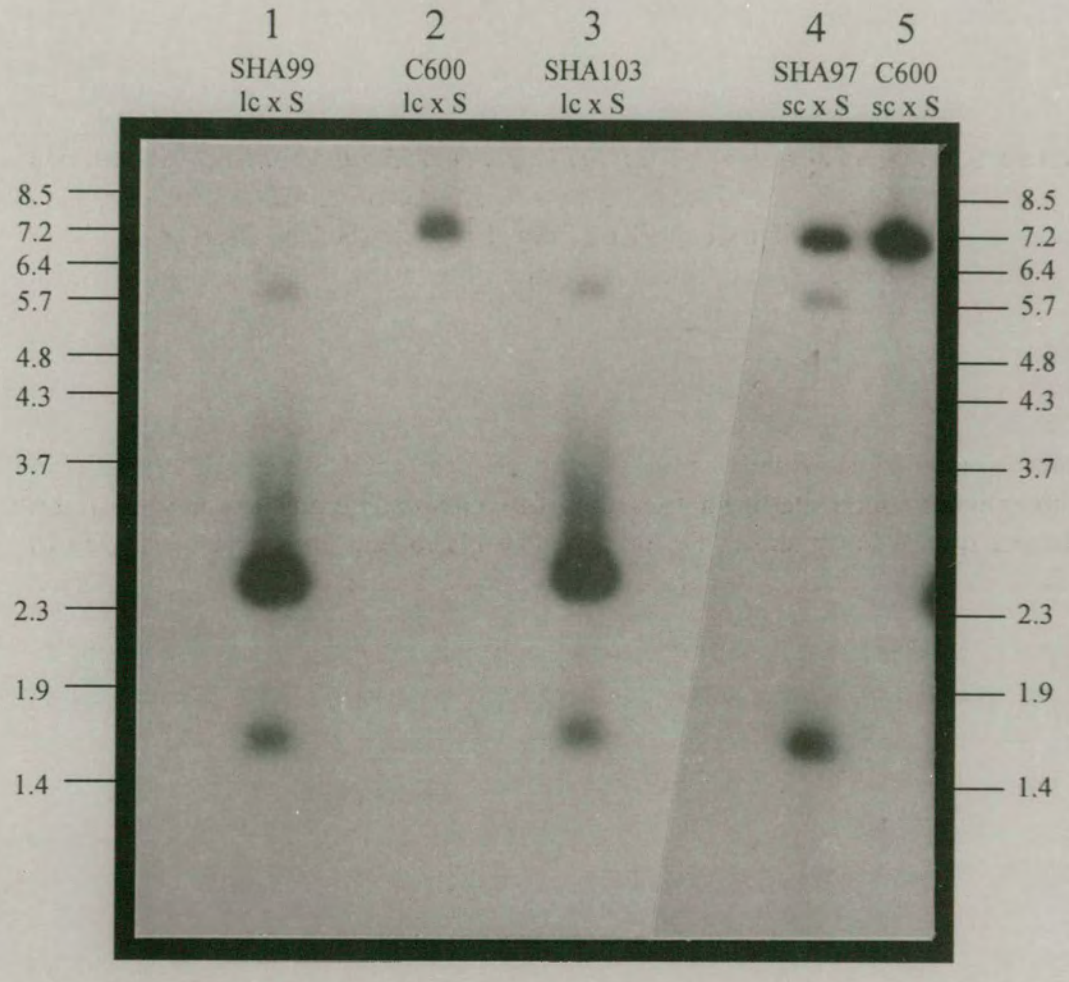
Further evidence for the lethality of orfUU disruptants. Phage P1 lysates were made on both strains and used for transductions into W3110 and W3110 pADD30. These recipients were transduced to Kan^R at 30°C. W3110 and W3110 pADD30 were easily transducible to Kan^R with the SHA103 lysate, however all transductants were also Cm^R showing that the plasmid had been integrated into the chromosome before the lysate was made. More interesting were transductions made with the SHA99 lysate. 47/47 transductants into W3110 pADD30 were Kan^R, Cm^S indicating that the chromosomal *orfUU*::Kan^R had been transduced without the complementing plasmid. When W3110 alone was used as the recipient, only five colonies were obtained. 4/5 were Kan^R, Cm^R and so had resulted from the transduction of the integrated plasmid along with the chromosomal marker. 1/5 was Kan^R, Cm^S and thus provided the

only evidence against disruption of *orfUU* being lethal. This strain (SHA97) was characterised along with the two *orfUU* disruptants in the following experiment, in order to resolve this anomaly.

Molecular confirmation of the disruption. In order to prove that the Kan^R gene was in fact on the chromosome of SHA103 and SHA99 and that it was definitely in *orfUU*, large-scale chromosomal DNA preparations (*2.2.12*) were made for Southern analysis. DNA from SHA97 was also prepared (small-scale, *2.2.12*) for the same experiment. One idea was that SHA97 might be a spontaneous Kan^R mutant and thus should show a wild-type chromosomal pattern around *orfUU*. As in section 5.4.5 restrictions were designed carefully so that chromosomal bands would not be obscured by brighter plasmid bands. A *Sal*I digestion of chromosomal DNA was probed with a PCR-amplified internal *orfUU* fragment, which straddled the *Bgl*II site used to disrupt the gene. If the insertion was on the chromosome, the probe was predicted to light up two fragments at 5.7 and 1.57 kb (due to *Sal*I sites in the cassette) together with a plasmid band of 2.4 kb. The wild-type chromosomal band would be easily separable from these bands at 7.27 kb (and if pADD56 or 57 were present, two plasmid bands at 1.57 and 1.1 kb would be expected).

Figure 5.4.6 shows the results of Southern analysis of SHA97, SHA103 and SHA99. Since previous results suggested that current colonies of SHA103 contained integrated plasmid, a new culture was inoculated from frozen stocks of the original colony. Controls in this experiment were C600 chromosomal DNA (both large and small-scale) restricted with *Sal*I.

Figure 5.4.6 *Southern analysis of SHA97, SHA103 and SHA99.* Restriction digests, agarose gel electrophoresis, radioactive labelling of probes, Southern blotting and hybridisation were all performed as described in Chapter 2. Filters were probed with a PCR-amplified, internal portion of *orfUU*, and separately with lambda DNA to detect the λcI^{857} x *Bst*EII markers. The latter are shown at the sides of each picture. Digests are labelled as follows: lc = large-scale chromosomal DNA, sc = small-scale chromosomal DNA, S = *Sal*I. In order to show the pattern of the two small-scale preparations, on the right-hand side of the filter, a longer autoradiographic exposure was necessary than for the rest of the filter. The figure is therefore a composite of photographs of two autoradiograms of the same filter. The gel quite clearly shows the predicted bands for chromosomal disruption of *orfUU* in both SHA103 and SHA99, compared to the wild-type C600 DNA. Plasmid bands are as predicted. The pattern of bands for SHA97 is very intriguing. It shows a characteristic wild-type band at 7.27 kb and two bands characteristic for the disruption at 5.7 and 1.57 kb. It shows no plasmid bands. This is discussed in the text.


1
SHA99
lc x S
2
C600
lc x S
3
SHA103
lc x S
4
SHA97
sc x S
5
C600
sc x S
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4

Southern analysis confirmed that the chromosomal *orfUU* was disrupted in strains SHA103 and SHA99. All the genetic experiments indicated that this disruption was lethal without a complementary plasmid, except for the appearance of SHA97, a strain which initially appeared to have inherited the disruption and survived without complementary DNA. The Southern analysis showed that SHA97 actually had two copies of the *orfUU* gene, one wild-type and one disrupted. The only explanation for this is that SHA97 contains a chromosomal duplication which includes *orfUU* and DNA up to the *Sal*I sites either side of it. This phenomenon of large chromosomal duplications was discussed at length in Chapter 3 and further characterisation of SHA97 is described below. Clearly, viable W3110 orfUU disruptants could only be isolated if either [i] the plasmid with the wild-type copy of the gene was also transduced, due to its integration into the chromosome (4/5 colonies) or [ii] if the recipient cell already had two copies of the chromosomal region around *orfUU* (1/4 colonies, SHA97). Table 5.4.2 shows that the integrant cells constitutes less than 6% of the population and although no data is available for the frequency at which the fifteen minute region DNA is duplicated in *E. coli*, markers at 7 minute and 20.5 minutes in the *S. typhimurium* chromosome were found to be duplicated at 0.035% and 0.008% respectively (Anderson and Roth, 1981). The latter statistic indicates that the former is probably an over-estimate. Taken together however, they both indicate that the five events recorded by the transduction into W3110 were very rare indeed. This is supported by the fact that none of the transductants into W3110 pADD30 were of either of these two types. This is a strong indication that *orfUU* is essential.

Genetic results, in conjunction with the Southern analysis, prove that disruption of *orfUU* is lethal to the cell. It can be concluded that *orfUU* is probably an essential gene. Two provisos remain: [i] because the whole of *orfU* is present on pADD23', the lethality could be due to a polar effect on *orfU* expression. If anything however, the strain with the Kan^R gene transcribing into the *orfU* gene (SHA103) was more unstable than that with the reverse (SHA99); [ii] although these results themselves are good evidence that *orfUU* is a gene, this has not been proven. There is a slim possibility that this open reading

frame is an untranscribed or untranslated region involved in *orfU* expression.

With this cautionary note in mind the phenotype of the *orfUU* disruption was investigated. Further experiments were also carried out on SHA97. These are described in the following two sections.

The phenotype of disrupted orfUU. A preliminary temperature shift experiment was carried out on SHA99 as described for SHA87 and SHA88 in section 5.4.5. The only features which were noticed were a gradual slowing of growth rate and cells appeared to get smaller. An experiment where SHA99 was inoculated at 44°C and its OD and morphology monitored is illustrated in Figure 5.4.7. It shows that decreased growth rate and reduced size at the non-permissive temperature was reproducible. The results are described in detail in the accompanying legend.

Figure 5.4.8 shows photographs of samples from the beginning and end of the 44°C growth curve in Figure 5.4.7 observed under conditions for visualisation of nucleoids (*2.3.5*). These samples showed that the phenotype did not include production of anucleate cells or any defect in nucleoid positioning, chromosome partitioning (by inference) or cell morphology, except for size. It was concluded therefore that the phenotype of a disruption of *orfUU*, was gradual decrease in cell size and ultimate cell-death for unknown reasons.

Figure 5.4.7 *Temperature shift experiment with SHA99.* The OD_{600} (■) and median cell-volume (○) of an SHA99 LB culture are shown. The culture was grown at 30°C until 175 min where it was diluted into prewarmed medium at 44°C. The culture was maintained in log phase throughout the experiment by keeping its OD_{600} between ~ 0.1 and 0.3.

The OD_{600} shows that the culture initially had a higher growth rate at 44°C than it had at 30°C. At about 325 min it began to slow down until about 400 min when the growth rate was below that of the initial 30°C culture. Subsequently the growth rate increased again. It appears that there was no actual cessation of growth at any time.

The median cell volume data shows that, after the dilution to 44°C, the cells reduced in size steadily, reaching about half their original size 250 min after the temperature shift. They then increased in size again to the end of the experiment (taking account of the fact that the last sample had an OD_{600} of ~1).

It is difficult to explain why when plating experiments showed a high degree of lethality at this temperature, there were no obvious indications of cell death. It may be that the resurgence of plasmid integrants came before the final death of the *orfUU*::Kan^R cells was observed. It could also be that the *orfUU* disrupted phenotyope is more severe on plates than in liquid. Nevertheless, the experiment described previously, where a 44°C culture was plated out for single colonies at 30°C, showed that only integrants and subsequently cured cells could survive in liquid at the non-permissive temperature.

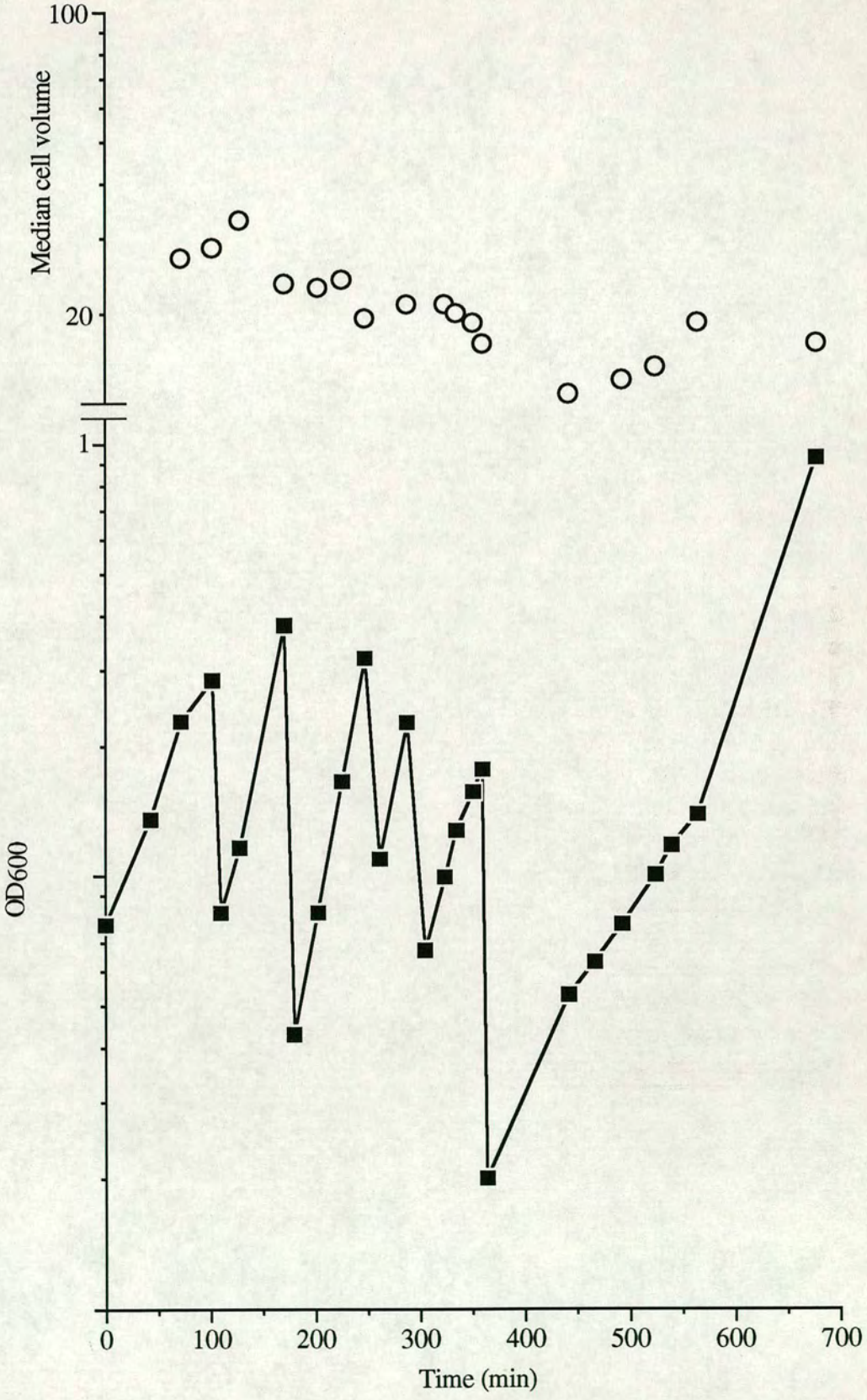
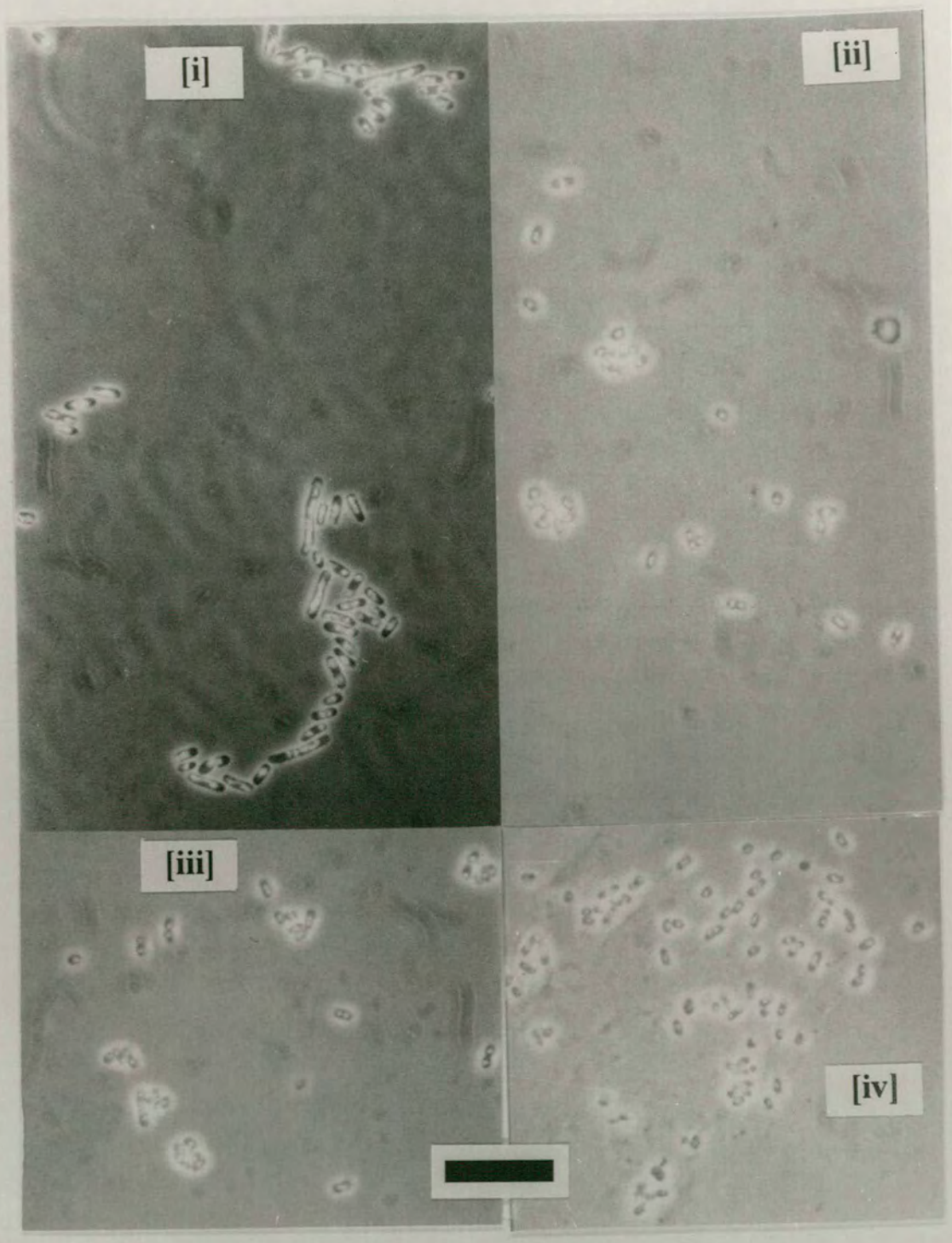

100
20
1
Median cell volume
OD600
0
100
200
300
400
500
600
700
Time (min)

Figure 5.4.8 *The orfUU phenotype.* Cells from the growth curve illustrated in Figure 5.4.7 were taken for microscopic analysis. Cells from the culture at 30°C (171 min) and 44°C (442 min) were taken for comparison. Because of the lower OD_{600} of the 44°C cells, the sample was concentrated before spotting on to the slide. The method for visualisation of nucleoids was used as described (*2.3.5*).

[i] shows the 30°C cells and [ii] – [iv] show the 44°C cells. The scale bar represents 10 μm. It can be seen that the only obvious phenotype of the disruption of *orfUU* is that the cells are much smaller than wild-type. The small size may be entirely due to the slowing of growth rate observed in Figure 5.4.7, although it was apparent from that figure that size decreased before growth rate was affected. The cells in [ii] – [iv] look very like cells entering or in stationary phase. This happened despite the low OD_{600} and fresh medium, indicating that OrfUU may be necessary for some general metabolic process, its inactivation leading to a phenotype reminiscent of starvation.


[i]
[ii]
[iii]
[iv]

Further characterisation of SHA97 - a partial diploid strain in the fifteen minute region. As a consequence of the results in Chapter 3 which identified a chromosomal duplication in the two minute region, it was proposed that this kind of strain could be used to make otherwise lethal chromosomal interruptions (disruptions or deletions)[11] and would be ideal for *in situ* single copy gene fusions which may otherwise be lethal. The discovery of SHA97 immediately validated the first idea. This approach has been used successfully in this laboratory for producing chromosomal disruptions of lethal genes in the two minute region (using SHA6, D Boyle, unpublished). Some ideas that were developed for the aforementioned experiments were applied to SHA97.

One of the major properties of the partial diploid strains was their instability. Therefore SHA97 was streaked out onto LB plates overnight and subsequent single colonies were screened for Kan^R. 1/40 colonies screened had become Kan^S (SHA102), confirming that SHA97 was unstable with a frequency of homozygotisation of the same order of magnitude as that of partial diploids described in Chapter 3. To further confirm the position of the *orfUU* disruption and the presence of the duplication, SHA97 was transduced to Tet^R using a phage P1 lysate of SHA76. This has a Tn*10* 88% cotransducible with *rodA* which is slightly closer to *orfUU*. SHA99 was a control for these transductions. 55/115 (55%) of the Tet^R transductants had become Kan^S for SHA97 whereas 42/60 (70%) became Kan^S for SHA99. This was further confirmation in both cases, that the cassette had correctly inserted into the chromosome and the lower cotransduction frequency in SHA97 supported the presence of a duplication. That is, in some cases the Tn*10* would have been transduced into the copy of that part of the chromosome which did not contain the disruption.

Two Tet^R, Kan^R SHA97 transductants were screened for instability and segregation of the two antibiotic resistances. It was hoped that they would have the Tet^R gene in the 'other' copy of the duplication from that which had the *orfUU* disruption (the most likely

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[11] This idea has been proposed independently by J Roth (personal communication) for *S. typhimurium*.

result in a colony with both resistances). These transductants were streaked overnight onto LB, LBT and LBK. Single colonies were classed according to their ability to grow when repatched onto the same media. The results are presented in Table 5.4.3.

Table 5.4.3 Segregation characteristics of partial diploids containing *orfUU*::Kan^R and Tn*10* on opposite duplicated regions.

			Phenotype:			
			Tet^R, Kan^R	Tet^R, Kan^S	Tet^S, Kan^R	Tet^S, Kan^S
Grown on:	LB	clone 1	24	1	0	0
		clone 2	20	5	0	0
		Total	44	6	0	0
	LBT	clone 1	24	1	0	0
		clone 2	21	4	0	0
		Total	45	5	0	0
	LBK	clone 1	25	0	0	0
		clone 2	25	0	0	0
		Total	50	0	0	0
			No. of colonies which grew:			

The results showed that [i] Tet^R, Kan^S segregants arose in the absence of Kan selection, but not when Kan was present. [ii] No reciprocal segregants were detected (Tet^S, Kan^R) even when there was only Kan present. These two observations suggested that the two markers were not on the same duplication, however segregation to a homozygous *orfUU*::Kan^R was lethal. This would explain why the number of segregants with and without Tet selection were approximately the same (10%) and would suggest that the actual segregation frequency was 20%. Repetitions of this experiment with colonies from these plates further confirmed that it was not possible to get a Tet^S segregant even with Kan selection. There is an alternative explanation for this segregation pattern which is that the region containing the transposon was not duplicated, hence no Tet^S segregants could appear by gene conversion. This however, is not supported by the cotransduction differences between SHA97 and

SHA99. This experiment therefore gave more evidence for the lethality of *orfUU* disruptions.

The Kan^S segregant of SHA97 which was initially detected (SHA102) was used as a possible homozygous partial diploid. A P1 lysate made on FI99 (*pbpA99* linked to Tn*10*) was used to transduce SHA102 to Tet^R and these transductants were screened for *ts*, the idea being that if the *pbpA* gene was also duplicated, no transductants would become *ts*. In a control transduction into C600 25/42 colonies were *ts* (60%). Transduction into SHA102 was poor but 2/7 transductants were ts. This was taken to mean either [i] the duplication in SHA97 and SHA102 did not include *pbpA*, or [ii] the SHA102 segregant had lost the duplication. A result described in the next paragraph indicated that [ii] was not the case. Given further time the extent of the duplication would have been ascertained in a similar way to that of SHA6 (*Chapter 3, Table 3.2.10*). The possibilities for using this strain and derivatives for chromosomal disruption of other genes in the fifteen minute region would then have been evaluated.

After initial isolation of SHA97 it was noticed that when, grown at 30°C, colonies contained a significant proportion of filamentous cells. At this time it was thought that SHA97 was an uncomplemented *orfUU* disruptant and that this was the phenotype. The strain was streaked at 42°C and found to grow much better with very few filaments at all. It was subsequently found that streaking at lower, rather than higher, temperature led to exacerbation of the phenotype. At 24°C, SHA97 not only grew slowly, as would be expected at that temperature, but there was a severe reduction in the number of colonies when compared to higher temperatures, indicating cell death. Surviving cells were extremely filamentous. When it was realised that SHA97 contained a duplication of the chromosome, the possibility arose that this cold-sensitive (*cs*) phenotype could be due to either the *orfUU* disruption complemented in single copy or the duplication itself. Therefore the growth of SHA97 (*orfUU*::Kan^R and duplication), SHA99 (*orfUU*::Kan^R only), SHA103 (*orfUU*::Kan^R only) and SHA102 (duplication only, possibly) was tested by streaking out at 24°C and 37°C on LB. Both SHA97 and SHA102 showed a severe reduction in the number of colonies at 24°C

in contrast to the other two strains which showed similar amounts of colonies at both temperatures. Thus it was concluded that the *cs* phenotype was associated with the duplication, not with the disruption, and that SHA102 *did* contain the disruption (see previous paragraph). The fact that the duplication of a region of the chromosome could lead to a *cs*, filamentous phenotype is interesting and warrants further investigation.

Conclusions. It can be concluded that disruption of the chromosomal copy of *orfUU* with the Kan^R cassette is lethal. That this is because *orfUU* is an essential gene is not completely proven but would seem the most likely interpretation. Further work is required to confirm this. The phenotype of an *orfUU* disruption is gradual decrease in growth rate and cell size. This a good simple description of cells entering stationary phase and the similarity could indicate many things, from aberrant stationary phase gene regulation to aberrant import of nutrients leading to starvation.

5.5 Discussion

The fifteen minute region has been subjected to extensive molecular and genetic characterisation. The identification of the protein product of a protein encoded by *orfU*, together with the significant similarity to a family of existing glycolytic enzymes (*Chapter 4*) is overwhelming evidence for it being a novel gene. This gene has been named *phpB*. Redundancy in genes for glycolytic and other metabolic enzymes is very common in *E. coli* (Riley, 1993). Further work will reveal whether this gene fits into that pattern.

Mutational analysis of the fifteen minute region led to the isolation of a probable cell division mutant, FO1165 (*ftsP*). Further study of this mutant including identification of the mutated gene and protein involved, should be very interesting. Its proximity to a group of cell morphology genes may not be a coincidence. The isolation of a partial diploid strain in the fifteen minute region with a cold-sensitive, filamentous phenotype may be connected to aberrant expression of the *ftsP* gene or it could indicate the presence of another as yet undiscovered cluster of cell division genes.

It remains to be seen if any genes from the *leuS*-operon are related in function to the *mrd*-operon genes, although the partial or slow block to cell division observed in *rlpB* disruption strains indicates a possible connection. Further characterisation of this gene and its product is required to determine if it is directly involved with cell division.

Finally, another probable essential gene was identified in the *leuS*-operon by construction of disruptions of the *orfUU* open reading frame. This has not been given a name so far because of its lack of similarity to any known sequences (*Chapter 4*) and the non-descript nature of its phenotype.

Chapter 6
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